Original Research

BAX gene over-expression via nucleofection to induce apoptosis in human lens epithelial cells

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Abstract

Despite significant advances in cataract surgery techniques, posterior capsule opacification (PCO) remains a common complication. In PCO, remaining epithelial cells cloud the lens capsule and impair postoperative vision. This *in vitro* study was designed to investigate the potential of a gene-based approach, specifically over-expression of the proapoptotic *BAX* gene, to prevent PCO. Human lens epithelial cells (HLECs) were transfected by nucleofection with a plasmid encoding a fusion protein of green fluorescent protein and human BAX. The expression levels of *BAX* and its antiapoptotic counterpart *BCL2* were determined by realtime reverse transcription polymerase chain reaction, Western blotting and immunofluorescence. *BAX* over-expression-induced cell death was analyzed by fluorescence-activated cell sorting using the Annexin V antibody. Fluorescence microscopy and transmission electron microscopy were used to assess changes in morphology and ultrastructure. Differential expression of the downstream apoptosis-related factor, caspase 3, was detected by Western blotting. Nucleofection efficiency was high (nearly 80%). *BAX*-transfected HLECs showed remarkably enhanced *BAX* gene expression and BAX:BCL2 ratio, but relatively little change in endogenous *BCL2* expression. *BAX* over-expression also led to significant cytotoxicity, induction of apoptosis-related characteristics and activation of caspase 3. In conclusion, our results indicate that *BAX* gene over-expression can trigger cell death in HLECs *via* an apoptotic pathway. Thus, *BAX* may be a promising candidate for human gene therapy to treat PCO.

Keywords: lens epithelial cell, apoptosis, gene transfer, BAX, nucleofection

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Introduction

Posterior capsule opacification (PCO) is one of the most common complications of cataract surgery. According to a recent meta-analysis, 28.4% of patients who underwent extracapsular cataract extraction developed visually significant PCO within five years of surgery.[1] In children, the incidence is almost 100% in cases where posterior capsulorhexis was not performed.[2] PCO is caused by proliferation, migration and metaplasia of remnant lens epithelial cells (LECs) on the capsule after cataract surgery,[3] which impairs vision and may require additional surgery.

The main treatment for PCO is laser capsulotomy. While this procedure is effective and painless, it is not without risks and is not feasible for treating patients who cannot be safely positioned, such as the mentally disabled and young children. Improvements in surgical techniques, design modification of the implanted intraocular lens and application of antiproliferative drugs to the surgery site have aimed to prevent PCO. While these efforts have decreased the incidence of PCO, they have not completely eradicated it, and a robust alternative therapy for preventing PCO remains to be developed.

Recently, gene therapy-based approaches for preventing PCO have produced promising results. These have focused mainly on suicide genes,[4–8] cell cycle genes[9–13] and cytokine genes,[14–15] each of which have been associated with particular drawbacks. *BAX* is a strong proapoptotic gene. The encoded BAX protein plays an important role in regulating apoptosis by activating the caspase signaling pathway and inducing mitochondrial release of apoptotic molecules, such as cytochrome *c*, into the cytosol. Therefore, when *BAX* is expressed at high concentrations, the cell suicide programme is activated, leading to cell death and clearance. Previous *in vitro* and *in vivo* studies have demonstrated that over-expression of *BAX* can effectively suppress tumor growth and significantly enhance the killing effects of radiotherapy or chemotherapy on tumor cells.[16–20]

In the study presented herein, we investigated the potential of a *BAX*-based gene therapy approach for treating PCO by using an *in vitro* system with human LECs (HLECs). The two major methods of gene delivery are viral vectors or

non-viral strategies. Virus recombinant vectors achieve high transduction efficiency and have been the most frequently used strategy for gene transfer experiments aimed at treating PCO. However, viral-based gene delivery suffers from several limitations, including the time-consuming and laborious methods required to produce recombinant vectors, the high level of safety required for handling and processing procedures and the associated laboratory costs, and limitation of insert size. In addition, human clinical trials have revealed a risk of immunogenic reactions to viral-based vectors.

For proapoptotic genes, such as *BAX*, use of a viral vector is precluded by the fact that expression of the proapoptotic gene in the packaging cell line inhibits proliferation, making it difficult to obtain a high titer of the virus vector.[21] Although a binary adenoviral vector system with regulatory components of the *GAL4* gene has been developed to overcome the difficulties in constructing viral vectors expressing high concentrations of apoptotic genes,[22] the method is complicated by the requirement for co-transfer of another viral vector expressing a transactivator. Thus, we considered non-viral methods to achieve gene transfer of the *BAX* gene.

Nucleofector technology is a non-viral transfection method based on electroporation. This technology uses cell type-specific electrical parameters and solutions to optimize transfection efficiency and decrease potential cytotoxic effects. Moreover, unlike any of the other non-viral transfection methods, the nucleofector technology directly transports the DNA into the cell nucleus, thereby allowing for expression of the transfected DNA to be detected shortly after nucleofection.[23] Therefore, we chose the nucleofection technique to examine the therapeutic potential of the *BAX* gene for PCO in HLECs.

Materials and methods

Cell culture

The HLEC line, SRA01/04,[24] was grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum (FBS), 800 U/mL penicillin and 1 mg/mL streptomycin. Cells were cultured at 37°C in 5% CO_2 and then split twice weekly using 0.25% trypsin. For the transfection experiments, passaged cells at 70–85% confluency were used.

Plasmid vectors

The reporter plasmid encoding green fluorescent protein (GFP), C3-EGFP, was a generous gift from Hongshen Guo at Fudan University (Shanghai, China). The GFP-Bax plasmid, C3-EGFP-Bax, which expressed a fusion protein of GFP and human BAX, was kindly provided by R J Youle at the National Institutes of Health (Bethesda, MD, USA).[25] Plasmid DNA was amplified in *Escherichia coli* strain JM109 and isolated using a plasmid DNA purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Isolated plasmid was dissolved under sterile conditions in phosphate-buffered saline (PBS) to a final concentration of 1 μg/μL. DNA quality and quantity were determined using an ultraviolet spectrophotometer ($A_{260}/A_{280} > 1.8$).

Gene transfection

Gene transfection was conducted with the Nucleofector™ instrument and accompanying reagent kit (Amaxa, Cologne, Germany). Briefly, 1×10^6 HLECs were suspended in 100 μL Cell Line Nucleofector™ Solution V at room temperature, followed by addition of 5 μg plasmid. The mixture of HLECs, Nucleofector™ solution and plasmid was transferred to a 1 mm electroporation cuvette, which was then inserted into the Nucleofector™ I and reacted by means of the automated transfection programme X-01. Immediately after transfection, 1000 μL of RMPI 1640 medium was added to the cuvette to reduce damage to the cells and the transfected cells were transferred to prewarmed medium, supplemented with 15% FBS and plated onto six-well culture plates. The transfection efficiency was determined by observing the GFP expression under an inverted fluorescence microscope (Leica, Heerbrugg, Switzerland).

Realtime reverse transcription polymerase chain reaction

Total RNA was extracted from cultured cells using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Two micrograms of total RNA were applied as template for cDNA synthesis with reagents from a first-strand cDNA synthesis kit (Shinegene Molecular Biotechnology Co., Shanghai, China). The real-time reverse transcription polymerase chain reaction (RT-PCR) was carried out using the Hot Start fluorescent PCR core reagent kit for SYBR green I (Applied Biosystems Inc., Carlsbad, CA, USA). Gene-specific primers were: *BAX*: forward 5′ CTTTTGCTTCAGGGTTTCATC 3′ and reverse 5′ ATCATCCTCTGCAGCTCCAT 3′; *BCL2*: forward 5′ GACTTCGCCGAGATGTCCAGC 3′ and reverse 5′ GCACCTACCCAGCCTCCGTTA 3′; *β-actin*: forward 5′ CTCCATCCTGGCCTCGCTGT 3′ and reverse 5′ GCTGTCACCTTCACCGTTCC 3′. The thermal cycling amplifications were performed on an FTC-2000 Sequence Detection System (Fengling Bio. Co., Shanghai, China) as follows: initial denaturation step of 94°C for four minutes, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 60°C for 25 s and extension at 72°C for 25 s. Detection of the fluorescent product was carried out at the end of each 72°C extension step. All collected data were normalized against the per-run detected expression of the β-actin housekeeping gene.

Western blot analysis

Western blot analysis was essentially performed as previously described.[26] Briefly, cells were harvested, washed twice in PBS and lysed. The cell lysates (50 mg of protein) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the separated proteins were transferred to a nitrocellulose membrane by the wet transfer method using a Bio-Rad electro-transfer apparatus

(Hercules, CA, USA). After blocking in 10% non-fat milk in Tris-buffered saline containing 0.2% Tween-20, the membrane was incubated with primary antibody (mouse monoclonal anti-BCL2, anti-BAX and rabbit polyclonal anti-CASP3; all from Sigma, St Louis, MO, USA), followed by an appropriate horseradish peroxidase-conjugated secondary antibody (Sigma). Immunoreactive bands were visualized by an enhanced chemiluminescence method (Tanon Science & Technology Co., Ltd, Shanghai, China) according to the manufacturer's instructions.

Immunofluorescent staining

Transfected HLECs grown on glass coverslips were incubated for 48 h, washed with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. The fixed cells were then permeabilized with 0.2% Triton X-100 for 30 min at 37°C, rinsed with PBS, preincubated with 10% goat serum and incubated with the respective primary antibody (1:500 dilution) overnight at 4°C. After extensive washing with PBS, the cells were incubated with CY3-conjugated secondary antibody (1:300; Sigma) for 60 min at 37°C in the dark. The coverslips were mounted with 90% glycerol and viewed under a fluorescent microscope.

Fluorescence-activated cell sorting

Nucleofected HLECs were plated onto six-well plates and incubated under standard conditions. After cells adhered to the plate, dead (floating) cells were removed by washing with fresh medium. At 18, 36, 42 and 60 h later, the cultures were subjected to fluorescence-activated cell sorting (FACS) analysis to detect cell surface markers of apoptosis. Briefly, both floating and adherent cells were harvested, pelleted by centrifugation (1500 rpm for 10 min) and re-suspended in 100 μL binding buffer. Then, the cells were incubated with phycoerythrin (PE)-labeled Annexin V (Gene Company Ltd, Hong Kong, China) in the dark at room temperature for 15 min. Cells were then applied to the FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Transfected cells expressing GFP emitted green fluorescence, while apoptotic or necrotic cells stained by Annexin V-PE emitted red fluorescence. Untransfected cells were used as a negative control.

Microscopic analysis and Hoechst staining

Nucleofected cells were plated onto six-well plates and cultured under standard conditions with a medium change after cells attached to the dish. Morphological examination of cells was performed using XDS-1B phase-contrast microscopy (COIC, Shanghai, China) or fluorescence microscopy (Leica). Nuclei were visualized using a Hoechst staining kit (Beyotime Institute of Biotechnology, Jiangsu, China) and following the manufacturer's instructions. In brief, cells were grown directly on glass coverslips for 48 h, incubated with fixing solution for 10 min, washed twice in PBS and stained by Hoechst 33258 for five minutes. After a final PBS wash, the coverslips were mounted and viewed under a fluorescent microscope.

Electron microscopy

Forty-eight hours after nucleofection, cells were harvested and fixed in 2.5% glutaraldehyde for two hours. After washing with 0.1 mol/L PBS, cells were postfixed for one hour in 1% OsO_4 and dehydrated through a graded series of ethanol alcohol solutions. Cells were infiltrated, embedded in Epon and polymerized at 70°C for 12 h. Ultrathin sections were mounted on formvar and carbon-coated single-slotted grids and stained with 2% uranyl acetate and lead citrate before examination under a transmission electron microscope (H-600; Hitachi, Tokyo, Japan).

Statistical analysis

Experiments were performed in tripliate. Intergroup differences were assessed by Student's *t*-test or non-parametric Wilcoxon-signed rank test using Stata 7.0 statistical software (Stata Corporation, College Station, TX, USA). For two-group comparisons at different time points, the analysis of variance test was used. A P value less than 0.05 was considered statistically significant.

Results

Transfection efficiency of nucleofection with C3-EGFP

To determine the efficiency of nucleofector-mediated gene transfer, *GFP* reporter gene expression in HLECs was observed under a fluorescent microscope 48 h after transfection. Some HLECs showed GFP fluorescence within two hours after transfection. After 48 h, the proportion of GFP-positive cells was 78.3% $\pm$ 17.5% (Figure 1). The fluorescence intensity of GFP-positive cells peaked 48–60 h after transfection. Many of the cells still showed GFP fluorescence after 72 h

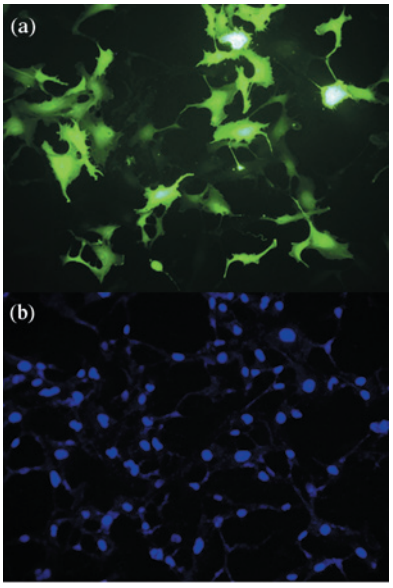

Figure 1 Photomicrographs of C3-EGFP (encoding green fluorescent protein)-nucleofected human lens epithelial cells after 48 h of culture. (a) Fluorescein isothiocyanate filters were used to visualize GFP-expressing cells. (b) Ultraviolet illumination shows 4′,6′-diamidino-2-phenylindole hydrochloride-stained nuclei of the corresponding cells. Magnification: ×200

in culture. These data demonstrate that nucleofector technology is capable of transferring exogenous genes to HLECs.

BAX and *BCL2* gene expressions after transfection with C3-EGFP-Bax or C3-EGFP

To determine the expression level of *BAX* and *BCL2* genes following transfections with the different plasmids, realtime RT-PCR and Western blotting analysis were performed. Expression of *BAX* and *BCL2* mRNAs was detected in *BAX*-transfected, *GFP*-transfected and untransfected HLECs using realtime RT-PCR. Meanwhile, the expression of BAX and BCL2 proteins was detected by Western blotting and immunofluorescent staining. BAX mRNA and protein concentrations were low in untransfected cells. In the *BAX*-transfected group, however, both BAX mRNA and protein expression were significantly enhanced (by 2.7- and 2.3-fold, respectively, $P < 0.05$) 48 h after *BAX* gene transfer, compared with cells transfected with C3-EGFP (Figures 2a and d). In contrast, the expression of BCL2 mRNA and protein in the *BAX*-transfected group was 97.7% and 60.1%, respectively, of that in the *GFP*-transfected group, but the difference was not statistically significant (Figures 2b and d). As a result, the BAX:BCL2 ratios of mRNA (2.7) and protein (3.8) were significantly elevated in the *BAX*-transfected cells, compared with *GFP*-transfected cells ($P < 0.05$) (Figures 2c and d).

Effects of *BAX* over-expression on cell death

To observe cell death following nucleofection, FACS analysis was carried out. We used the Annexin V-PE cell membrane labeling assay to detect translocation of phosphatidylserine from the inner face of the plasma membrane to the cell surface, where it binds an Annexin V-PE conjugate and serves as a marker of apoptosis or necrosis. Successfully transfected cells expressing GFP emitted green fluorescence and apoptotic or necrotic cells stained by Annexin V-PE emitted red fluorescence. After *BAX* gene transfer, the cell death rate in successfully transfected cells was approximately 70%, which was significantly higher than that in cells transfected with C3-EGFP (30%, $P < 0.05$). No significant difference was observed in the cell death rate at different time points (Figure 3).

Ability of *BAX* to induce apoptosis

To examine the mechanism of *BAX*-induced cell death, we analyzed known apoptosis biomarkers by microscopy (i.e. structural features) and Western blotting (i.e. caspase 3 levels). Changes in cell morphology were examined by phase contrast microscopy and fluorescence microscopy, while changes in nuclear morphology were evaluated by Hoechst 33258 staining. Changes in cellular ultrastructure were assessed by transmission electron microscopy. After *BAX* gene transfer, *BAX*-transfected cells presented with morphological features of poor growth, including a fuzzy profile, cellular shrinkage (Figures 4a and c), chromatin condensation and margination (Figure 4e), and nuclear fragmentation (Figure 4g), compared with *GFP*-transfected cells (Figures 4b, d, f and h). In addition, the *BAX*-transfected cells had significantly higher concentrations of caspase 3 expression (by 2.4-fold, $P < 0.05$) than *GFP*-transfected

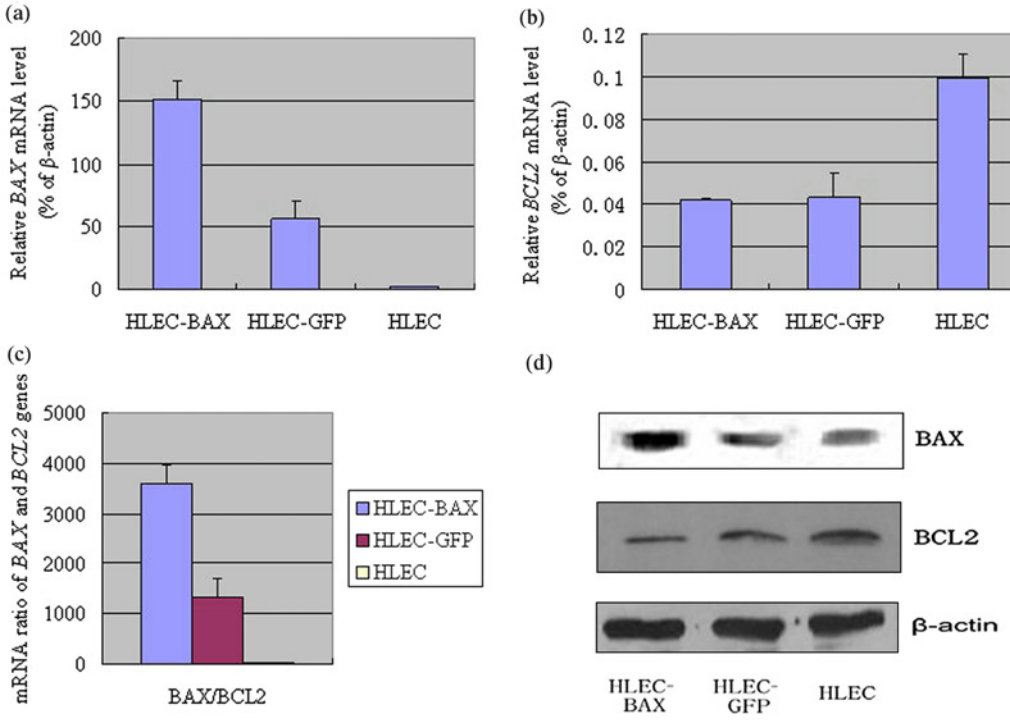

Figure 2 Gene expression in *BAX*-transfected, green fluorescent protein (*GFP*)-transfected and untransfected human lens epithelial cells (HLECs) at 48 h after nucleofection. (a) mRNA concentrations of *BAX* gene, detected by realtime reverse transcription polymerase chain reaction. (b) mRNA concentrations of *BCL2* gene. (c) The mRNA ratio of *BAX* and *BCL2* genes. (d) BAX and BCL2 protein expression, detected by Western blot analysis. (A color version of this figure is available in the online journal)

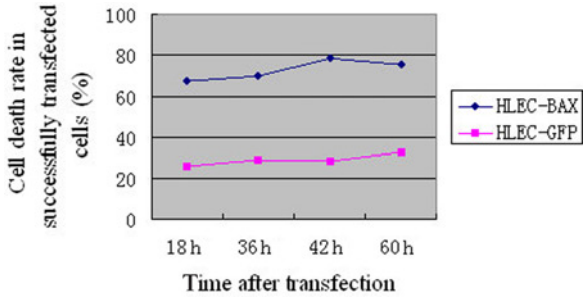

Figure 3 Cell death rate in successfully *BAX*-transfected cells and green fluorescent protein (*GFP*)-transfected cells. Cells were analyzed by a flow cytometer 18, 36, 42 and 60 h after transfection. Transfected cells expressing GFP emitted green fluorescence, while apoptotic or necrotic cells stained by Annexin V-phycoerythrin emitted red fluorescence. Cell death rate was significantly different between the two groups. (A color version of this figure is available in the online journal)

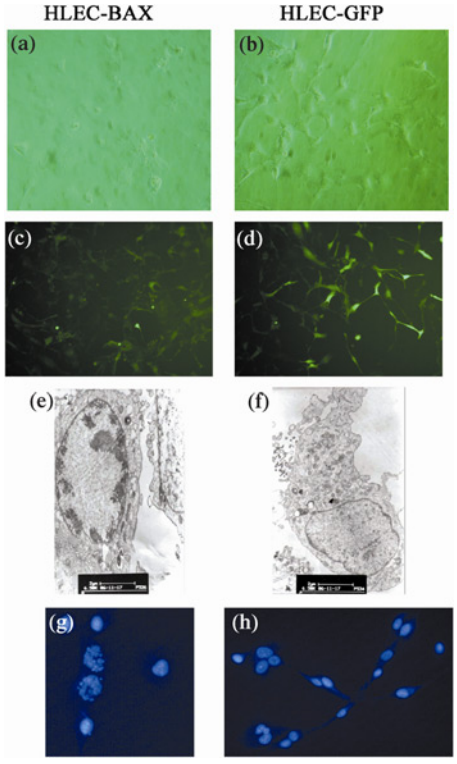

Figure 4 Morphology study of human lens epithelial cells (HLECs) after transfection with C3-EGFP (encoding green fluorescent protein)-Bax (left column) or C3-EGFP (right column). (a, b) Phase contrast microscopy of HLECs 48 h after transfection (×250). (c, d) Fluorescent microscopy of HLECs 64 h after transfection (×100). (e, f) Electron microscopy of HLECs 48 h after transfection (bar: 2 μm). (g, h) Hoechst staining of HLECs 48 h after transfection (×200)

cells (Figure 5). Collectively, the results suggested that cells underwent death *via* the apoptosis pathway.

Discussion

Here, we describe a gene transfer technique using nucleofector technology to over-express the proapoptotic *BAX* gene in HLECs. The nucleofection strategy achieved transfection efficiencies of up to 78%, and GFP-positive cells were detected as early as two hours after nucleofection.

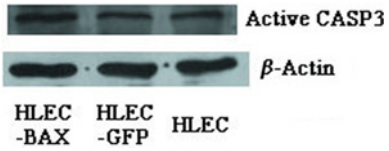

Figure 5 Caspase 3 protein expression in *BAX*-transfected, green fluorescent protein (*GFP*)-transfected and untransfected human lens epithelial cells (HLECs) 48 h after nucleofection, as detected by Western blot analysis. (A color version of this figure is available in the online journal)

Importantly, the transfection efficiencies in our experiments were comparable with those reported for viral-based gene transfer systems. For example, Liu *et al.*[10] and Sun *et al.*[11] transduced HLECs with adenoviral vectors and achieved efficiencies of 75–80%, while Malecaze *et al.*[4] reported an efficiency of 95% in primary rabbit LECs. Using retroviral vectors, Couderc *et al.*[5] achieved transfection efficiencies of only 29% in the primary rabbit LECs. Therefore, nucleofector technology is capable of producing similar transfection efficiency to the commonly used but more complex viral-based methods in epithelial lens cells.

The potential of gene therapy for PCO has been investigated for more than ten years, and the majority of studies have focused on suicide genes, cell cycle genes and cytokine genes. However, all of the gene therapies developed to date have inherent flaws. The suicide gene-based methods use enzyme-encoding genes that are non-toxic in isolation, but which can catalyze the formation of highly toxic metabolites in the presence of a significantly less toxic prodrug.[4–8] As such, the suicide gene therapy induces necrosis, which inevitably arouses a potentially detrimental inflammatory response. Cell cycle gene therapy modulates the expression of cell cycle control genes, such as *p21, cyclin G1* and *MAT1*, and induces cell cycle arrest in the G1 or G2 phase by down-regulating the expression of cyclins or inhibiting the activity of the cyclin-dependent kinase complex.[9–13] Therefore, whenever the exogenous gene expression decreases below a certain threshold or vanishes completely, the cells can re-enter mitosis. Cytokine-related gene therapy down-regulates the expression of some cytokines, such as basic fibroblast growth factor, in order to inhibit cell proliferation.[14–15] Thus, like the cell cycle gene therapy, this method requires stable and long-lasting expression of exogenous genes.

To overcome the limitations of each of the previously developed gene-based approaches listed above, we selected *BAX*, a key proapoptotic gene, for our study. After *BAX* gene transfer, the cell death rate in successfully transfected cells was approximately 70%, which was significantly higher than that in cells transfected with empty plasmid. Morphology studies showed that *BAX*-transfected cells developed many morphological features of cell death. Finally, the *BAX*-transfected cells also had significantly enhanced caspase 3 protein levels. All of these characteristics suggested that cells with *BAX* over-expression underwent death *via* the apoptosis pathway.

The results also showed that compared with untransfected cells, cells transfected with C3-EGFP also had an elevated BAX concentration, a decreased BCL2 concentration and an approximate cell death rate of 30%. We ascribed

these effects to the circular plasmid DNA that was transfected. Seiffert *et al.* reported that the nucleofection-based strategy could achieve high transfection efficiency and cell viability in B cells and the B cell chronic lymphocytic leukemia (B-CLL) cell line by using a special co-culture system. Surprisingly, the introduction of circular plasmid DNA induced rapid apoptosis, which was independent of the type of transgene used but dependent on the DNA concentration. Further experiments proved that only transfer of circular plasmid DNA, but not linear DNA or RNA molecules, induced apoptosis in transfected B cells or B-CLL cells.[27] It is likely that the HLECs in our study possess a similar process leading to apoptosis after cytoplasmic accumulation of foreign DNA. One possible mechanism underlying this may even be the elevated BAX/BCL2 ratio.

This newly developed proapoptotic gene therapy approach is superior to the previous gene therapies in several regards. First, apoptosis is the predominant mechanism of *BAX* over-expression-induced cell death in LECs, rather than necrosis and will not arouse an inflammatory reaction in the body. Second, instead of maintaining a quiescent state (i.e. inhibiting cells from entering into mitosis), *BAX* over-expression induces cell death and the affected LECs should be cleared from the body; this process eliminates the possibility of cells overcoming the exogenous gene expression and re-entering the proliferation process. Lastly, *BAX* over-expression led to cell death within a very short period of time, eliminating the need for stable expression of the exogenous proapoptotic gene and decreasing the complexity of the gene transfer procedure. All of these features support the notion that *BAX* may be a promising candidate for human gene therapy of PCO.

Since modern-day cataract surgery has evolved to such a safe and effective procedure, the requirements for PCO preventive therapies include a high level of safety and convenience for application in the clinical setting. One concern about the nucleofector-based technique described herein is its use of electric fields and their potential effects *in vivo*. However, *in vivo* electroporation, using proper electric parameters, has been demonstrated by several studies to be safe in organs and tissues *in situ*, including in the eyes.[28–30] Another concern of this gene therapy strategy is the use of a proapoptotic gene. As a strong proapoptotic gene, intraocular application of *BAX* may induce the death of normal cells, such as corneal endothelial cells. However, the directivity of electroporation may be exploited to overcome this problem. When applied *in vivo*, a ring-shaped positive electrode can be placed on the ocular surface above the lens equator, and a small ball-shaped negative electrode can be placed in the center of the capsular bag after lens removal. In this manner, the electrical field will drive the plasmids towards the positive electrode (i.e. the lens equator, where the residual LECs exist), helping to ensure that very few corneal endothelial cells will be affected. Such a methodological design could physically facilitate targeted gene transfer in the lens.

While our newly developed nucleofector-based proapoptosis gene therapy has produced promising results *in vitro*, further *in vivo* studies are required to test the efficacy and safety of this technique for treating PCO.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. YF conducted the experiments and wrote the manuscript; and YL gave critical revisions to the manuscript.

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