

Growth inhibition of human lens epithelial cells by short hairpin RNA in transcription factor forkhead box E3 (FOXE3)

Ye Wang · Wenfeng Li · Yao Wang · Yusen Huang

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Abstract

Background Posterior capsule opacification occurs mainly due to the remnant lens epithelial cell proliferation and migration after cataract surgery. The purpose of this study was to investigate whether small hairpin RNA (shRNA)-mediated gene silencing of transcription factor forkhead box E3 (FOXE3) can be employed to inhibit the expression of FOXE3 and suppress the growth in lens epithelial cells.

Methods FOXE3-targeted shRNA was transfected into a human lens epithelial cell line (HLEB-3) using Lipofectamine 2000 reagent. Quantitative PCR was used to confirm the downregulation of FOXE3 mRNA expression following infection of lens epithelial cells, and FOXE3 protein expression levels were evaluated by Western blot analysis and immunofluorescence staining. HLEB-3 cell growth after the transduction was analyzed by cell counting and MTT colorimetric assay. Cell cycle of the HLEB-3 cells was examined by flowcytometric analysis.

Results Compared with the control groups, both mRNA and protein levels of FOXE3 expression were significantly decreased in shRNA-treated groups, and cytostatic effects were obvious within 48 h after transfection. An increased

incidence of G1-phase arrest was identified in FOXE3-shRNA transfected HLEB-3 cells.

Conclusions shRNA-mediated gene silencing of FOXE3 could significantly inhibit cell growth and induce the G1-phase arrest in HLEB-3 cells. Formation of posterior capsular opacification might be repressed if lens epithelial cell growth ceases after the FOXE3 gene is silenced with molecular biology technology.

Keywords Posterior capsular opacification · FOXE3 gene · shRNA

Introduction

Posterior capsular opacification (PCO), also termed secondary cataract, is the most frequent complication of cataract surgery [1]. Advances in surgical techniques and intraocular lens materials and designs have reduced the rate of PCO. However, it is still a significant clinical problem and is even more threatening in young adults and children, with a higher incidence, earlier onset, and greater amblyogenic effect [2, 3]. Lens epithelial cells (LECs) left behind in the capsular bag after any type of extracapsular cataract surgery are mainly responsible for PCO development [4]. Proliferation, migration, epithelial-to-mesenchymal transition (EMT), collagen deposition, and lens fiber regeneration of LECs are the main causes of opacification.

FOXE3/FREAC-8/FKHL12, first described by Larsson and co-workers in 1995, (accession numbers for human sequences: Nucleotide NM_012186, Protein AAF82793) is a lens-specific member of the large forkhead transcription factor family. FOXE3 encodes a DNA-binding transcription factor whose expression is coincidental with the formation

Ye Wang and Wenfeng Li contributed equally to this work

Y. Wang · Y. Wang · Y. Huang (✉)
State Key Laboratory Cultivation Base,
Shandong Provincial Key Laboratory of Ophthalmology,
Shandong Eye Institute, Shandong Academy of Medical Sciences,
Qingdao 266071, China
e-mail: huang_yusen@126.com

W. Li
Department of Oncology,
Affiliated Hospital of Qingdao University Medical College,
Qingdao, China

of the lens placode [5, 6] and also in the testis and prostate. The expression and function of FOXE3 were conserved in vertebrates [7]. During development, FOXE3 transcripts are present in the developing lens. In adult mice, expression is limited to the anterior lens epithelium [5, 6]. FOXE3 expression was also found in the eye and brain of zebrafish embryos, with the highest levels in lens epithelial cells, and FOXE3 transcripts were also detected in the adult lens and brain [8]. In human embryo coronal head sections (Carnegie stage 16 and 17), FOXE3 expression was found in the developing lens only, with the strongest expression in the anterior lens epithelium [9]. Its expression is initially detected in the lens-forming surface ectoderm after contact with the optic vesicle (murine E9.5), and is maintained there during lens placode formation, invagination, and lens vesicle separation from the overlying ectoderm. When primary lens fiber differentiation occurs, FOXE3 expression becomes confined to the lens epithelium.

FOXE3 was an obvious candidate gene for developmental lens defects, and FOXE3 is essential for closure of the lens vesicle. FOXE3 is also involved in the maintenance of central lens epithelial cell proliferation via the upregulation of Mki67, Pcn1 and Pdgfra expression, and the maintenance of equatorial lens epithelial cell phenotype via the exclusion of Prox1-induced Cdkn1c expression [5]. This prevents early cell cycle exit and premature lens epithelial cell differentiation into lens fiber cells. In mice with targeted deletion of both alleles of FOXE3 [10], the cells of the anterior lens epithelium cease to proliferate prematurely, and the lens is smaller and is sometimes almost absent. Proper inactivation of FOXE3 expression at the lens equator is important for many aspects of fiber differentiation, and persistent expression leads to partial epithelialization of fiber cells [11].

Our previous study found that FOXE3 expression is only detected in the lens epithelium during rat PCO formation [12]. Because overgrowth of LECs onto the posterior face of the lens capsule is the cause of PCO, formation of PCO might be repressed if LEC growth ceases after the FOXE3 gene is silenced with molecular biology technology. Short-chain hairpin RNA (shRNA), which is synthesized by *in vitro* transcription, strongly inhibits gene expression and has high sequence specificity. Short-chain hairpin RNA (shRNA) can block the expression of genes rapidly and continuously [13]. Based on the above understanding of shRNA, we will use liposome, which has higher efficiency and smaller adverse reaction as a reagent to seed FOXE3-shRNA and transfect it into the lens epithelial cells. In this study, expression of FOXE3 and interference with the efficiency of FOXE3-shRNA in lens epithelial proliferation were investigated. We hope to provide a theoretical basis for an *in vivo* experiment.

Materials and methods

Reagents

Opti-MEM® I medium was purchased from Invitrogen Corporation (Carlsbad, CA, USA). Thiazole (MTT, methyl thiazolyl tetrazolium) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). For immunofluorescence, immunoblotting, and staining procedures, the following antibodies were used: goat polyclonal anti-FOXE3 (Abcam, London, UK), secondary antibody (mouse anti-goat-horseradish peroxidase; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; KangChen-BioTech, Shanghai, China).

shRNA construction

Three target sequences of shRNA were designed according to the FOXE3 sequence in the GenBank (NM_012186) according to the rules of Tuschl. We designed three small hairpin RNA (shRNA) sequences (F1, F2, and F3) and a nonsense sequence (HK). As shown in Table 1, the hairpin RNA constructs were made by a unique 19-nt double-stranded human FOXE3 sequence that is an inverted complementary repeat and is separated by a loop of 9-nt spacer (underlined). The shRNA products were constructed from Genesil Biotechnology Co., Ltd (Wuhan, China). The HK sequence served as a negative control.

Cell culture and treatment

Human lens epithelial B-3 (HLEB-3) cells (a transformed human lens epithelial cell line [14], kindly provided by Dr. Chen Youhai, the University of Pennsylvania Gene Therapy Laboratory) was maintained in Opti-MEM® I medium supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% CO₂. For FOXE3 gene expression assay and immunofluorescence, HLEB-3 cells were seeded in 24-well plates at a density of 1.5×10^5 cells per well. For proliferation and apoptosis assays, HLEB-3 cells were seeded in six-well plates at a density of 4×10^4 cells per well. The cells were allowed to grow to 60% confluence. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. The concentrations of shRNA were 50 nmol/l, 100 nmol/l, and 200 nmol/l. Cells transfected with HK and liposome were used as negative controls. The vehicle control group was transfected with liposome without shRNA. The blank control group was transfected with the same quantity of Opti-MEM® I medium.

Table 1 Oligonucleotide sequence of FOXE3 specific shRNA

Name	Sequence of shRNA
F1 ^a	5'-TCATCACCGAACGATTTGCTTGAAGAAGGCAAAGCGTTCGGTGATGATT-3'
F2 ^a	5'-CCGAACGCTTTGCCTTCTTTGAAGAAGTAGAAGGCAAAGCCTTCGGTT-3'
F3 ^a	5'- GACTTCATAAGGCGCATGC TTGAAGAAG GCATGCGCCTTATGAAGT TT-3'
(HK) ^b	5'-GACTTCATAAGGCGCATGCTTGAAGAAGGCATGCGCCTTATGAAGTC TT-3'

^a F1, F2 and F3, three small hairpin RNA (shRNA) sequences (F1, F2, and F3) of FOXE3 gene

^b HK, nonsense sequence (HK) of FOXE3 gene

Underlining indicates loop of 9-nt spacer

Western blotting analysis

Cells were lysed in a buffer containing 50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), and 1% TritonX-100. Total protein in cell lysates was measured with the Bio-Rad colorimetric kit. After SDS-PAGE on 12% linear slab gel, separated proteins were transferred to a polyvinylidene fluoride membrane using a semidry electrophoretic transfer cell (Trans-Blot; Bio-Rad, Richmond, CA, USA). After blocking in a 10% nonfat dry milk solution in washing buffer containing 10 mmol/l Tris (pH 7.6), 150 mmol/l NaCl and 0.05% Tween-20, membranes were incubated for 1 hour at room temperature with goat anti-human FOXE3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then they were washed 4 times and incubated for another hour with goat anti-mouse horseradish peroxidase (HRP)-coupled secondary rabbit anti-goat antibodies (Zhongshan Golden Bridge Biotechnology, Beijing, China) at room temperature. Signals were detected with the ECL kit (Amershan Pharmacia, UK). GAPDH staining served as the internal standard for the membrane. Obtained images were digitized. Densitometry was performed by computing the relative density of each shRNA band to a GAPDH band of the same sample using Digital Science ID Image Analysis software.

Real-time PCR

Real-time PCR was performed with the TaqMan probe for FOXE3. Primers used for amplification of the FOXE3 gene were as follows: forward primer 5'-TCATCACCGAACGCTTTGC-3' and reverse primer 5'-GGGCACCTTGACGAA GCA-3'. Primers used for amplification of the GAPDH gene were as follows: forward primer 5'- CCACTCCT CCACCTTTGAC-3' and reverse primer 5'- ACCCTGT TGCTGTAGCCA-3'. The TaqMan probe was designed using Primer Express Software (Applied Biosystems). The sequence of the FOXE3 TaqMan probe was 5'-CCC GCG CAAGTGGCAGAACA-3'. The sequence of the GAPDH TaqMan probe was 5'-TTGCCCTCAACGACCACTTT GTC-3'. For the TaqMan probe method, the probe was

labeled at the 5' end with reporter dye FAM and at the 3' end with quencher dye TAMRA. Real-time PCR was performed using the FTC2000 (Funglyn Biotech, Toronto, Ontario, Canada). Samples were set up in 30 µL final volumes containing 6 µl 5× PCR buffer, 0.6 µl 2× primers (25 pmol/µl), 0.6 µl probe (25 pmol/µl), 1 µl dNTPs (10 mM), 0.3 µl Taq enzyme (5 U/µl), 3 µl Mg²⁺ (25 mM), 1 µl template, and 16.9 µl DEPC water (Sigma-Aldrich, St Louis, MO, USA). Amplification conditions consisted of 4 min at 94°C, followed by forty 20-s cycles at 94°C and finally 30 s at 60°C. The relative expression was calculated based on the expression of GAPDH.

Immunofluorescence

Immunofluorescence was performed 48 h after transfection. Cells grown on glass coverslips were placed in 1:1 acetone: methanol pre-chilled to -20°C and incubated for 10 min or longer at -20°C. The cells were blocked for 1 h with blocking solution (10% normal serum in PBS), incubated for 2 h with polyclonal anti-FOXE3 (Abcam, London, UK) at a 1:50 dilution, washed with PBS, incubated for 40 min with rabbit anti-goat (Tianjin Haoyang Biologic Manufacture, Tianjin, China) at a 1:400 dilution, washed with PBS again and mounted with UltraCruzTM mounting medium (Santa Cruz Biotechnology, Santa Cruz, CA). The cells were counterstained with DAPI (Molecular Probes, Eugene, OR, USA) to visualize nuclei. For negative controls, secondary antibodies alone were used without a primary antibody treatment. Cells were photographed with a Leica fluorescence microscope (Leica Microsystems AG Wetzlar, Germany).

Cell growth assays

Cell inhibition rate was assayed through a colorimetric 3-4,5-(dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay 48 h after transfection at a concentration of 100 nmol/l in 96-well plates. The cells were incubated at 37°C for 4 h after the MTT solution (5 mg/ml, Sigma). After incubation, dimethyl sulfoxide (DMSO, Nacalai Tesque,

Japan) 150 μ l/well was used instead of the MTT solution. The plates were shaken on a microshaker for 10 min before the absorbances were examined at a wavelength of 550 nm using a microtiter plate reader (Labsystems Oy, Helsinki, Finland). Nontransfected HLEB-3 cells were used as a control group. The above procedure was performed three times. The cell inhibition rate was calculated as follows: [(A550 of control group–A550 of experimental group)/A550 of control group] \times 100%. A550 means absorbance of 550 nm.

The cytostatic effect of shRNA was also evaluated by counting the number of viable cells in each culture. HLEB-3 cells were transfected with shRNA on the second day after being seeded at a density of 4×10^4 cells/well in six-well plates. Cell number was recorded at 24, 48, and 72 h after transfection. HK-shRNA transfected HLEB-3 cells were the control group. The above procedure was performed three times.

Flowcytometric analysis

HLEB-3 cells were collected 48 h after transduction with shRNA, centrifuged at 1,500 rpm for 5 min and washed with 3 ml ice-cold phosphate balanced solution (PBS) by centrifugation at 1,500 rpm for 5 min. Then the cells were gently suspended in 100 μ l ice-cold annexin V incubation reagent (apoptosis detection kit, Beckman Coulter, France) containing 2.5 μ l propidium iodide (PI), 5 μ l annexin V-FITC (annexin V conjugated to FITC) and $1 \times$ binding buffer before incubation in the dark at 4°C for 10 min. Ice-cold $1 \times$ binding buffer (150 μ l) was added to each sample, which was analyzed using flow cytometry (Beckman Coulter, Miami, FL, USA) with MultiGraph software within 1 h to obtain the maximum signal.

Annexin V-FITC binding to PS indicated apoptotic cells; PI staining was used to identify cells that lost membrane integrity and were classified as necrotic rather than apoptotic. Cells positive for annexin V-FITC fluorescence alone were identified as apoptotic, whereas cells positive for both PI fluorescence and annexin V-FITC fluorescence were identified as necrotic. Annexin V FITC was excited with a wavelength of 488 nm light, and had an emission peak at 515 m, while propidium iodide was detected at 650 when excited with 488 nm wavelength light. Cells stained by PI were detectable in the FL2 channel, and apoptotic cells recognized by annexin V-FITC were detectable in the FL1 channel.

Cell cycle status in the transfected cells was determined by measuring nuclear DNA content. HLEB-3 cells were collected at 48 h after transfection with shRNA, centrifuged at 1,500 rpm for 5 min and washed twice with 3 ml ice-cold PBS by centrifugation at 1,500 rpm for 5 min. The cells were fixed in 70% ethanol at 4°C for more than 4 h. The pellet was collected by centrifugation at 1,500 rpm for 5 min and washed twice with 3 ml ice-cold PBS before the RNase solution was

added (final concentration 50 μ g/ml). After 1 h of incubation at 37°C, the cells were stained with PI (final concentration 100 μ g/ml) at 37°C for 30 min. Flow cytometry was used to analyze the samples with Multi-Cycle software.

Statistical analysis

Results are expressed as mean \pm SD. Statistical significance was determined by the independent-samples *t*-test, and differences at $p < 0.05$ were considered statistically significant.

Results

FOXE3 protein inhibition in HLEC cells by Western blotting

The expression of FOXE3 protein was studied by semi-quantitative Western blot analysis. A representative immunoblot for FOXE3 in HLEB-3 cells is shown in Fig. 1a. A sharp decrease in FOXE3 protein levels was detected 48 hours after transfection (Fig. 1b). Densitometry analysis with Image J showed that these groups of different concentrations of 50 nmol/l, 100 nmol/l, and 200 nmol/l compared with control groups were significantly different (all $p < 0.05$), and the relative density ratios (FOXE3 band to GAPDH band) of FOXE3 protein were 0.385, 0.713 and 0.722 respectively. However, the negative group and the vehicle group compared with the blank control group were not significantly different ($p > 0.05$) 48 h after transfection.

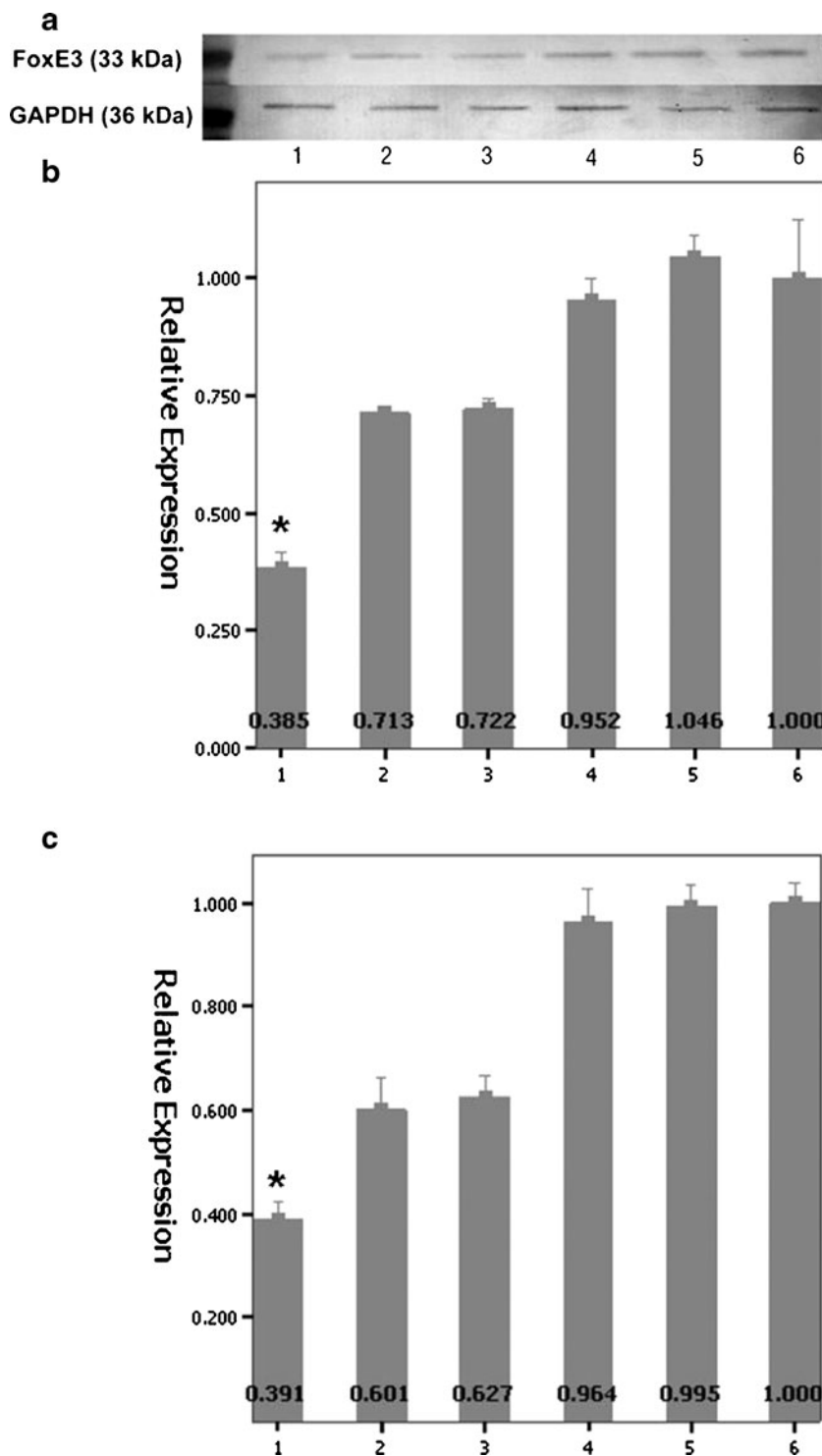
FOXE3 mRNA inhibition in HLEC cells by PCR

Real-time PCR analysis revealed that FOXE3 mRNA expression was significantly decreased 48 h after transfection (Fig. 1c). The concentration of 50 nmol/l, 100 nmol/l, and 200 nmol/l were significantly different from control groups (all $p < 0.05$). However, the negative control (HK-shRNA) and vehicle control (liposome group) groups were not significantly different from the blank control group ($p > 0.05$). The FOXE3 mRNA expression levels at concentrations of 50 nmol/l, 100 nmol/l, and 200 nmol/l were decreased by 60.93%, 39.95% and 37.32% respectively, compared with the blank control. Of the concentrations tested, 50 nmol/l is the best. These results showed that FOXE3 mRNA expression was specifically decreased by F1-shRNA.

FOXE3 protein inhibition in HLEB-3 cells by immunofluorescence staining

In Fig. 2, protein expression of FOXE3 in the blank control group and the HK-shRNA group was stronger than that of the F1-shRNA group. Cell density and fluorescence

Fig. 1 FOXE3 expression of HLEB-3 cells. **a** Western blot analysis of FOXE3 protein compared to GAPDH as the internal control. **b** Relative expression of FOXE3 in different groups according to Western blot analysis. A significantly lower level of survivin protein was detected in cells transfected with shRNA. The concentration at 50 nmol/l was most effective. **c** Real-time PCR showed that the relative expression of FOXE3 was lower in FOXE3-shRNA transfected HLEB-3 cells than in control groups, which is consistent with the Western blot analysis. Lane 1, 50 nmol/l of FOXE3-shRNA transfection; lane 2, 100 nmol/l FOXE3-shRNA transfection; lane 3, 200 nmol/l FOXE3-shRNA transfection; lane 4, non-targeting RNA transfection (negative control); lane 5, vehicle control; lane 6, blank control. * Significantly different from the control groups and other shRNA groups ($p < 0.05$)



intensity of FOXE3 decreased significantly 48 h after transfection with F1-shRNA.

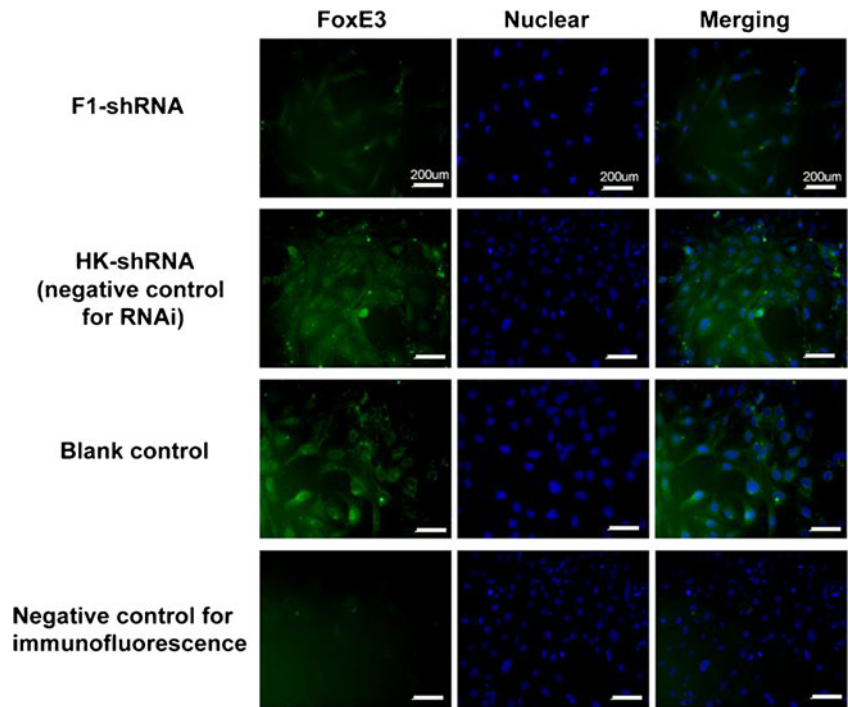
Cell growth inhibition, apoptosis and cycle in the shRNA-transfected HLE B-3 cells

There was an obvious decrease in the number of viable HLEB-3 cells from 24 to 72 h after transfection. At the

end of the observation period (72 h), there were 16.08×10^4 HLEB-3 cells in the F1-shRNA group (100 nmol/l) (Fig. 3a), much less than in the nontransfected group (48.25×10^4 cells) ($p = 0.000$).

The FOXE3-shRNA-transfected HLEB-3 cells shrank 24 h after transduction and detached from the incubation plates at 48 h and 72 h. Cell density also fell below the starting value (Fig. 3d). After transfection for 48 h and

Fig. 2 Immunofluorescence staining of FOXE3 in HLEB-3 cells. Forty-eight hours after transfection with shRNA, the cells were fixed for immunostaining. FOXE3 protein expression of the FOXE3-shRNA-transfected group was decreased in comparison to control groups. The *green signal* represents staining of the FOXE3 proteins, and the *blue signal* represents nuclear DNA staining by DAPI. Bar size is 200 μ m



72 h, cells exposed to the negative control group and the blank control group had normal epithelial morphology (Fig. 3b and c).

Data of MTT colorimetric assay were shown in Table 2. The absorbances were 0.351 ± 0.000 , 0.503 ± 0.032 , 0.525 ± 0.016 , 0.745 ± 0.023 , 0.782 ± 0.037 , and 0.784 ± 0.015 in the F1-shRNA group, F2-shRNA group, F3-shRNA group, HK-shRNA group, vehicle control group and blank control group at 48 h respectively. The absorbances of the F1-

shRNA group and other groups were significantly different (all $p < 0.05$), and the absorbances of the HK-shRNA group, vehicle control group and blank control group were not significantly different (all $p > 0.05$). There were fewer viable cells of F1-shRNA group than other groups. The percentage of viable cells were 47.11%, 67.52% and 70.47% in F1-shRNA group, F2-shRNA group and F3-shRNA group respectively. Therefore, the suppression rate of cells were 52.89%, 32.48% and 29.53% in F1-shRNA group, F2-

Fig. 3 The change of morphology of HLEB-3 cells transfected with FOXE3-shRNA. HLEB-3 cells shrunk after 48 h transfection with FOXE3-shRNA and detached from the plates (a), while the cells exposed to the negative (b) or blank (c) control groups had normal epithelial morphology. The cell growth curve shows the number of HLEB-3 cells in the FOXE3-shRNA transfected group compared with the negative control group (d). Cell proliferation is expressed as cell number (vertical axis) over time (horizontal axis). The bars represent the standard deviation of the mean cell number. Bar size is 50 μ m

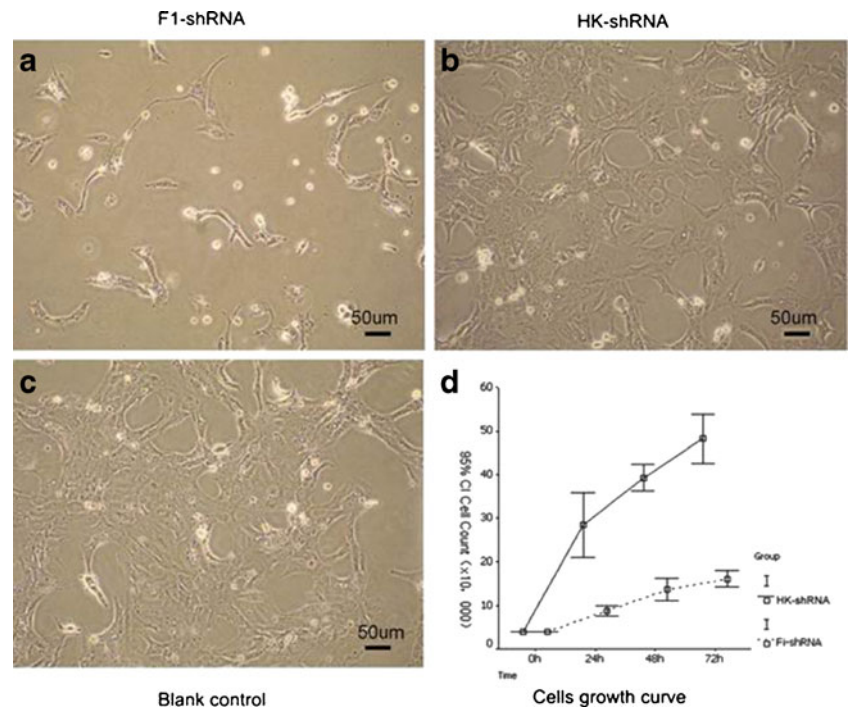


Table 2 Data for the MTT colorimetric assay

Groups	Absorbance	Cell inhibition rate ^b
F1-shRNA	0.351±0.000 ^a	52.89%
F2-shRNA	0.503±0.032	32.48%
F3-shRNA	0.525±0.016	29.53%
HK-shRNA	0.745±0.023	
Vehicle control group	0.782±0.037	
Blank control group	0.784±0.015	

Values are means ± SD

^aSignificantly different from the control groups and other shRNA groups ($p < 0.05$)

^bThe cell inhibition rate was calculated as follows: [(A550 of control group–A550 of experimental group)/A550 of control group] × 100%. A550 means absorbance of 550 nm

shRNA group and F3-shRNA group respectively. These data indicated that HLEB-3 cell proliferation was inhibited most significantly in the cultures transfected with F1-shRNA.

It has previously been reported that FOXE3 is essential for closure of the lens vesicle, and promotes survival and proliferation, and FOXE3 mutation can induce apoptosis in mice [5]. So we next studied apoptosis and the cycle of transfected HLEB-3 cells. An increased incidence of apoptosis was noticed in the F1-shRNA transfected HLEB-3 cells. The ratio of apoptotic cells to total cells was $12.0 \pm 1.33\%$ 48 h after F1-shRNA transduction, much higher than $0.18 \pm 0.01\%$ in the HK-shRNA group ($p = 0.002$) and $0.12 \pm 0.02\%$ in the blank control group ($p = 0.004$) (Fig. 4).

G1-phase arrest occurred after transduction with F1-shRNA, manifested as an increase of cell percentage in G0/G1 phase ($45.85 \pm 0.83\%$) and a decrease in S phase ($20.17 \pm 4.50\%$). Also, a change of cell percentage in G2/M was observed ($33.97 \pm 4.25\%$) after F1-shRNA transfection (Table 3). Statistical analysis showed significant differences

Table 3 Cell cycle status of the HLE B-3 cells

Groups	Cell percentage (%)		
	G ₀ / G ₁	S phase	G ₂ / M
F1-shRNA	45.85±0.83	20.17±4.50	33.97±4.25
HK-shRNA	39.09±0.47	36.20±0.57	24.71±0.47
Blank control group	39.69±0.81	40.41±3.71	19.90±3.00
<i>P</i>	0.0009	0.0079	0.0030

HLEB-3 cells were fixed and stained with PI. DNA content was quantified by flow cytometry. G1-phase arrest occurred after transduction with F1-shRNA, manifested as an increase of cell percentage in G0/G1 phase ($45.85 \pm 0.83\%$) and a decrease in S phase ($20.17 \pm 4.50\%$). Also, a change of cell percentage in G2/M was observed ($33.97 \pm 4.25\%$) after F1-shRNA transfection

between the transduced group and the negative-control or the blank-control group in both G₁% and S% at 48 h ($p < 0.05$) after transduction.

Discussion

Transcription factors regulate the spatio-temporal expression of thousands of genes, ensuring the proper development and function of the organism. Various transcription factors, expressed in defined subsets of cells, can act to modulate a series of signals to produce specific cell types. During eye development, the timely action of transcription factors and inductive signals ensure the correct development of different eye components. FOXE3 has high sequence-conservation and is expressed in zebrafish, mouse, and human LECs [13]. FOXE3 is essential for proliferation, prevention of premature differentiation, and protection against apoptosis in the lens epithelium.

In a previous study, we showed that FOXE3 protein is only detected in lens epithelial cells during rat PCO formation

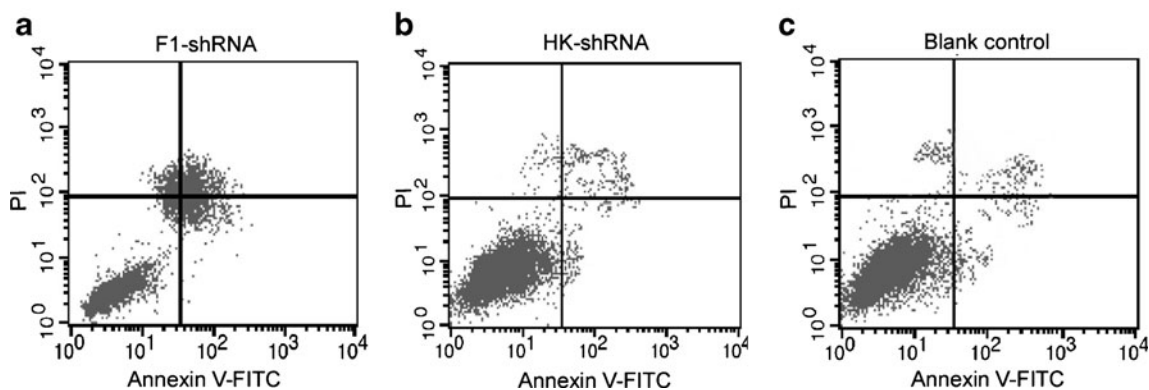


Fig. 4 Cell apoptosis induced by FOXE3-shRNA transfection. Two-parameter dot plots of flow cytometric analysis on HLEB-3 cells after staining with Annexin V- FITC and propidium iodide (PI). **a** FOXE3-shRNA transfected HLEB-3 cells at 48 h. **b** HK- shRNA transfected

HLEB-3 cells at 48 h. **c** Blank control group. The ratio of apoptotic cells to total cells was $12.0 \pm 1.33\%$ 48 h after F1-shRNA transduction (**a**), much higher than $0.18 \pm 0.01\%$ in the HK-shRNA group ($p = 0.002$) (**b**) and $0.12 \pm 0.02\%$ in the blank control group ($p = 0.004$) (**c**)

by immunofluorescence [12]. Because FOXE3 is essential for proliferation of LECs, we presume that PCO may be depressed or lessened if its expression is inhibited. To verify our deduction, we used RNA interference to study the effect of FOXE3 silencing in the proliferation of cells in vitro. In the present study, we found that the expression of the FOXE3 gene could be depressed specifically in mRNA and protein, and the proliferation of HLECs was also inhibited. Thus, we can expect to use RNA interference aimed directly at the FOXE3 gene as a tool in the depression of PCO in vivo.

As the purpose of our study is to suppress the expression of FOXE3 rapidly and to observe the effect of RNA interference and gene silencing in cellular growth inhibition, we selected HLEB-3 cells and used liposome, which has higher efficiency as a reagent to transfect cells with biosynthesized shRNA directly. The HLEB-3 cells, which are human fetal lens epithelial cells transfected with adenovirus Ad-12 and simian virus SV40, have characteristics of human origin, normal epithelial cell morphology, and normal expression of lens epithelial cell specific proteins, α - and/or β -crystallin, they are widely used as a model for in vivo studies of HLE cell physiology, eye-related toxicology, and cataracts [15–17]. In this study, we find that this cell line can also express FOXE3 gene and can be as a useful tool for related studies. One study used human epithelial cell primary culture to study the effect of RNA interference [18]. Although their physiological characteristics may be more similar to in vivo conditions than a human lens epithelial cell line, there are two shortcomings of this method. First, it takes lens epithelium 10 days to expand to 70%–80% confluence, which is much longer than cell lines. Second, it is difficult to transfect primary epithelial cells using standard plasmid-based transfection techniques. This problem is resolved by using lentiviral vectors to express shRNA constructs [18].

As a rule, it is best to use the lowest concentration of shRNA that produces maximal silencing of the intended target. In the current study, we observed dose-dependent inhibition of mRNA and protein with shRNA treatment in HLEB-3 cells. Target gene silencing was detectable when the shRNA concentrations were 50 nmol/l, 100 nmol/l, and 200 nmol/l. Of the concentrations tested, 50 nmol/l is the best, indicating mRNA and protein expression of the FOXE3 gene of more than 60%. Higher shRNA levels might be toxic to normal HLEB-3 cells or off-target effects [19]. These results indicate that F1-shRNA at a concentration of 50 nmol/l or less selectively inhibits FOXE3 gene expression in HLEB-3 cells.

This study also found that shRNA-mediated gene silencing of FOXE3 can lead to apoptosis of HLEB-3 cells. Inhibition of HLEB-3 cells growth is partly due to apoptosis. In a normal developing lens, a distinct border separates the dividing cells of the epithelium from the posterior lens and differentiates cells, and this border coincides with the

limit for FOXE3 expression. The gene encoding forkhead transcription factor FOXE3 is expressed in the lens epithelium, and is turned off when fiber differentiation begins. In FOXE3-null mutants, the lens epithelium gradually disappears during embryonic development. The result is a small lens with severe cataract that fails to separate from the cornea [5, 6, 20]. Mechanisms behind disappearance of the lens epithelium involve failure to proliferate, apoptosis, and premature onset of fiber differentiation. The *dyl* mutant lens has many apoptotic cells in the anterior part of the epithelium, whereas no signs of cell death can be detected in the wild-type lens [5].

We concluded that specific shRNA interference aimed at the FOXE3 gene can inhibit the expression of FOXE3, depress the proliferation of HLEB-3 cells, and induce apoptosis. Due to the tissue specificity of the FOXE3 gene and its function in the maintenance of LEC proliferation and prevention of premature differentiation and apoptosis, the shRNA-mediated gene silencing of FOXE3 is an attractive gene therapy for PCO in vivo. While this work is aimed at detecting the efficacy, in the following study, we will immerge further into the mechanism of the cell growth inhibition following FOXE3 knockdown, and in vivo confirmation is warranted.

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