• Basic Research •

Role of reactive oxygen species in epithelial-mesenchymal transition and apoptosis of human lens epithelial cells

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Abstract

● **AIM:** To investigate the role of reactive oxygen species (ROS) in epithelial-mesenchymal transition (EMT) and apoptosis of human lens epithelial cells (HLECs).

● **METHODS:** Flow cytometry was used to assess ROS production after transforming growth factor β2 (TGF-β2) induction. Apoptosis of HLECs after H_2O_2 and TGF-β2 interference with or without ROS scavenger N-acetylcysteine (NAC) were assessed by flow cytometry. The corresponding protein expression levels of the EMT marker α-smooth muscle actin (α-SMA), the extracellular matrix (ECM), marker fibronectin (Fn), and apoptosis-associated proteins were detected by using Western blotting in the presence of an ROS scavenger (NAC). Wound-healing and Transwell assays were used to assess the migration capability of HLECs.

● **RESULTS:** TGF-β2 stimulates ROS production within 8h in HLECs. Additionally, TGF-β2 induced HLECs cell apoptosis, EMT/ECM synthesis protein markers expression, and pro-apoptotic proteins production; nonetheless, NAC treatment prevented these responses. Similarly, TGF-β2 promoted HLECs cell migration, whereas NAC inhibited cell migration. We further determined that although ROS initiated apoptosis, it only induced the accumulation of the EMT marker α-SMA protein, but not COL-1 or Fn.

● **CONCLUSION:** ROS contribute to TGF-β2-induced EMT/ECM synthesis and cell apoptosis of HLECs; however, ROS alone are not sufficient for EMT/ECM synthesis.

● **KEYWORDS:** human lens epithelial cells; epithelial-mesenchymal transition; transforming growth factor β2; reactive oxygen species; apoptosis
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INTRODUCTION

Posterior capsular opacification (PCO) is the most common postoperative complication following cataract extraction surgery[1]. Recent studies have suggested that PCO is caused by classical fibrotic processes, including residual lens epithelial cell proliferation, transdifferentiation, migration, collagen production, and regenerative processes, such as lens fiber cell differentiation[1-2]. In our previous study, we determined that cataract surgery led to the recruitment of transforming growth factor β2 (TGF-β2) and promoted epithelial–mesenchymal transition (EMT), extracellular matrix (ECM) synthesis, residual lens cell migration, and cell apoptosis during PCO development[3]. However, the corresponding upstream regulatory pathways remain unclear. Further, the participating molecules and microenvironmental processes underlying the pathogenesis of PCO remain to be elucidated.

NOX proteins are catalytic subunits of the NADPH oxidase complex, which is located in nonphagocytic cells; specifically, they function as oxygen sensors and catalyze the reduction of molecular oxygen to reactive oxygen species (ROS)[4]. NOX4 has been identified as the major initiator of TGF-β-dependent ROS production, and subsequent oxidative stress, in PCO; consequently, NOX4 inhibition has been observed to impede the EMT process[5]. Interestingly, during treatment with TGF-β, NOX4 is first localized in the nucleus and then co-localizes with α-smooth muscle actin (α-SMA) in human lens epithelial cells (HLECs)[5]. Moreover, ROS can accelerate TGF-β activation, and TGF-β promotes NOX4 transcription, forming a feedback loop in fibrotic organs[6-7]. In pancreatic cancer cells, ROS may inhibit tumor development; this has been suggested ROS are suppressed by *Kras*, *Braf*, and *Myc* oncogenes[8]. In renal tubulointerstitial fibrosis, ROS-mediated TGF-β1 activates the p38 and MAPK signaling pathways[9].

However, in lens epithelial cells, the functional role of ROS within the PCO mechanism of action remains unclear, especially regarding its relationship with apoptosis.

In this study, we first assessed ROS production in HLECs stimulated by TGF-β2; then, we observed the effects of an ROS scavenger following TGF-β2 treatment; finally, we used H_2O_2 as an ROS source and elucidated the direct ROS-dependent induction on cell apoptosis and EMT/ECM synthesis.

MATERIALS AND METHODS

HLECs Culturing and Treatment HLEC line was purchased from American Type Culture Collection (CRL-11421) and was used for several experiments[3,10-11]. These cells were cultured in 1 g/L glucose Dulbecco's modified Eagle medium (DMEM; CM10014, MACGENE) containing 10% fetal bovine serum (FBS; 04-001-1ACS, Biological Industries) and penicillin–streptomycin (SV30010, HyClone) at 37℃ in a humidified atmosphere. Cells before passage 12 were used for all experiments in this study. The corresponding medium was replaced every alternate day. For cell treatment, N-acetylcysteine (NAC; A-9165-5G, Sigma-Aldrich) was added to the medium at a final concentration of 10 μmol/L to scavenge ROS.

Western Blot Analysis Western blotting was performed as previously described[11-12]. After 48h of TGF-β2 treatment with or without NAC treatment, the medium was discarded, and the cells were washed twice with phosphate buffer saline (PBS). Laemmli sample buffer (1610737, Bio-Rad) was then added to the wells and whole-cell lysates were harvested into Eppendorf tubes. Next, 20 μL samples were loaded into each lane for 10% polyacrylamide gel electrophoresis, then transferred onto polyvinylidene fluoridemembranes (PVDF). After incubation in bovine serum albumin/Tris-buffered saline with 0.1% Tween 20 detergent (BSA/TBST) solution at room temperature for 1h to blocking various proteins, PVDF membranes were incubated with primary antibodies against fibronectin (Fn; Sc-9068, Santa Cruz), type 1 collagen (COL-1) (ab138492, Abcam), α-SMA (A2547, Sigma), Caspase-3 (#9664, CST), Bcl-2 (ab32124, Abcam), Bax (#2772, CST), and Actin (#4970, CST), which were diluted in TBST, and placed at 4℃ overnight with gentle shaking. After thoroughly cleaning with TBST for 30min, the membranes were incubated with goat anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase at room temperature for 1h. Finally, images were taken using an enhanced chemiluminescence Western blotting detection kit (1705060; Bio-Rad).

Cell Apoptosis Detection by Flow Cytometry HLEC apoptosis was tested using an APC-PE-PI apoptosis assay kit (BioLegend) with a FLEX system (Beckman Coulter). Following TGF-β2 treatment with or without NAC or 200 μmol/L H_2O_2 treatment for different durations (0, 8, 12, 16, 20, or 24h), cells were collected and washed in Eppendorf tubes; HLECs were then resuspended in 100 μL of Annexin V binding buffer containing 5 μL of Annexin V and 10 μL of propidium iodide. After incubation at room temperature for 15min in the dark, 400 μL of binding buffer was added to terminate the reaction; cell samples were then analyzed using a flow cytometry FLEX system.

ROS Detection After induction with H_2O_2 for different durations (0, 4, 8, or 12h), 10 μmol/L DCFH-DA (Cat: D6883, Sigma-Aldrich) was added to the medium; these plates were then incubated at 37℃ for 12min in the dark. The cells were subsequently washed and resuspended in PBS. The fluorescence intensity was quantified using a flow cytometry FLEX system. Untreated HLECs were used as a blank, and positive cell frequency was used in statistical analysis.

Cell Migration and Invasion Evaluation We conducted wound-healing and Transwell assays to estimate the migration and invasion capability of HLECs treated with TGF-β2, with or without NAC, according to our previous protocol[10]. Briefly, for the Transwell assay, 10^4 cells were seeded in the upper chamber, and 600 μL of complete medium was placed in each well. When NAC was added to these wells, the corresponding FBS amount was adjusted to 1%; TGF-β2 was then added after 2h of pre-treatment. Following 24h of treatment, the chamber was stained with crystal violet and the cells that migrated through the membrane were counted under a microscope. In the wound-healing assay, 3×10^4 cells were seeded into the upper well of the insert, purchased from ibidi GmbH (Martinsried, Germany), which possesses a 500 μm space. When the insert was removed, the cells could move from each side into the gap. The insert was removed 24h later when each well was 90% confluent; then, after 2h of NAC pretreatment, TGF-β2 was added to induce cell migration. The cells were then incubated in medium containing 1% FBS for 24h. We collected images daily and performed statistical analysis using Image J.

Statistical Analysis All statistical analyses were performed using GraphPad Prism 6.0 software. Data are presented as the mean±standard error of the mean, taken from three independent experiments. We performed comparisons between two groups using Student's *t* test and between multiple groups using a one-way ANOVA. The results were considered statistically significant when the *P* value was <0.05.

RESULTS

Effects of TGF-β2 on ROS Production in HLECs Recent studies have shown that in renal fibrosis disease, TGF-β can enhance the expression of NOX4 and initiate NOX4-induced ROS-regulated TGF-β/Smad signaling in a feed-forward manner[6]. Similarly, ROS have been found to emerge in rat lens epithelial explants with PCO and aggravate EMT/ECM

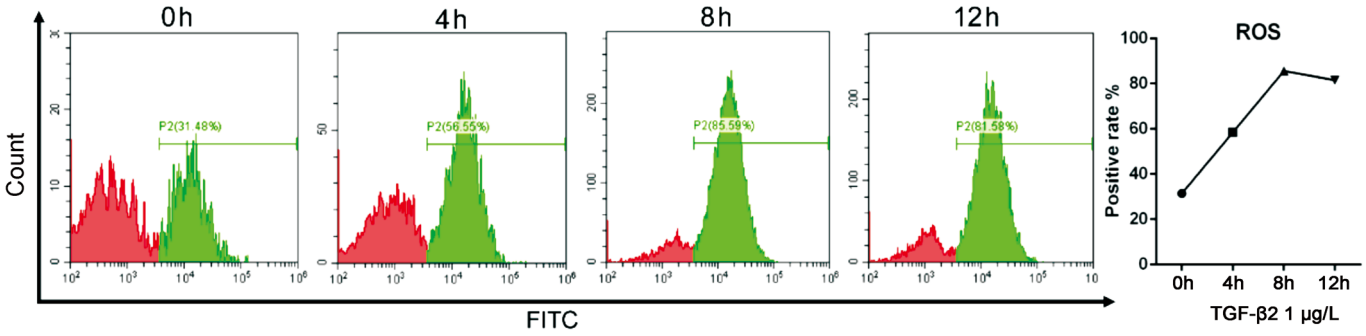

Figure 1 TGF-β2-induced ROS production HLECs were treated with 1 μg/L TGF-β2 across different durations (0, 4, 8, or 12h); ROS expression was measured by flow cytometry. TGF-β2: Transforming growth factor β2; ROS: Reactive oxygen species; HLECs: Human lens epithelial cells.

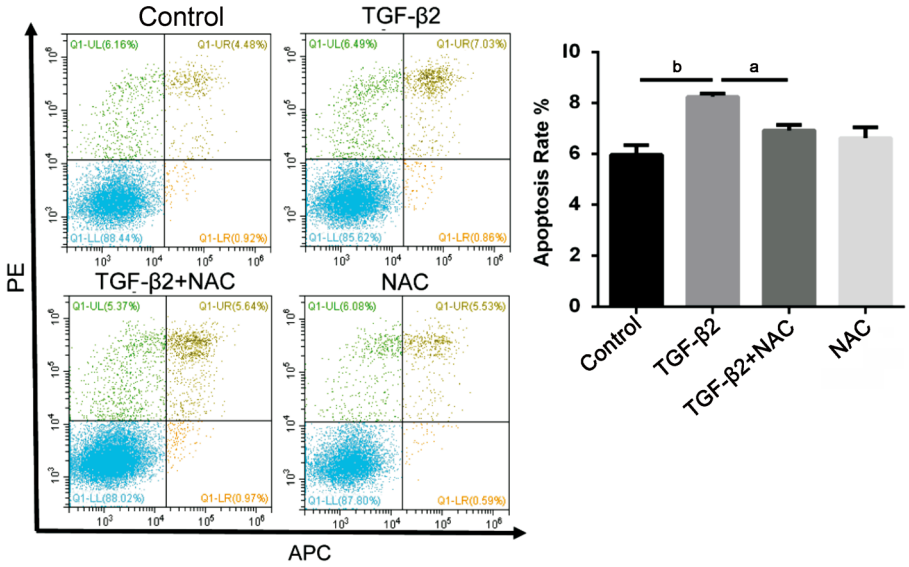

Figure 2 ROS mediate TGF-β2-induced HLECs apoptosis HLECs were treated with 1 μg/L TGF-β2 for 48h with or without 10 μmol/L NAC; cell apoptosis was detected by flow cytometry. Apoptosis rate was analyzed using a one-way ANOVA and Tukey's test for multiple comparisons. $^{a}P<0.05$, $^{b}P<0.01$. ROS: Reactive oxygen species; TGF-β2: Transforming growth factor β2; HLEC: Human lens epithelial cells.

synthesis[5]. TGF-β2 is specifically expressed in the lens and is widely used to mimic PCO conditions in molecular studies[2]. To confirm the specific role of ROS in PCO, we first detected whether TGF-β2 could induce ROS production in HLECs. By evaluating these samples using a flow cytometry system, we determined that TGF-β2 treatment led to an accumulation of relatively high levels of ROS after 8h of induction, which were determined to be maintained 12h after induction (Figure 1). Therefore, we verified that there may be relatively high ROS accumulation in the residual lens epithelial cells during PCO progression.

Effects of ROS Scavenging on Cell Apoptosis in HLECs We previously established that mitochondria-released ROS can activate the endogenous apoptotic pathway in HLECs during cataract progression[11]. We have also confirmed that lens epithelial cell apoptosis occurs following cataract surgery and functions as a mediator that promotes EMT/ECM synthesis during PCO progression[3]. Hence, we aimed to determine how ROS function in PCO cell apoptosis. We detected HLECs apoptosis by flow cytometry with TGF-β2 analysis. These demonstrated showed that a TGF-β2 concentration as low as 1 μg/L could induce significant cell apoptosis, especially late-stage apoptosis (Figure 2). In contrast, ROS scavenger (NAC) addition significantly reduced TGF-β2-induced HLECs apoptosis (Figure 2). Therefore, ROS were determined to activate HLECs apoptosis, and ROS scavengers could effectively prevent this TGF-β2-induced apoptosis. Thus, apoptosis of HLECs during PCO may be caused by ROS accumulation.

ROS Function in Cell Apoptosis and EMT/ECM Synthesis Since we confirmed that TGF-β2 could induce HLEC apoptosis in PCO, we aimed to determine whether ROS mediated the two major factors of PCO: cell apoptosis and EMT/ECM synthesis. The corresponding results demonstrated that TGF-β2 treatment increased the expression of not only fibrosis protein markers (α-SMA, COL-1, and Fn) expression, but also pro-apoptotic protein markers (cleaved Caspase-3 and Bax; Figure 3). In addition, when NAC treatment was introduced, to remove ROS from these cells, the expression of both the fibrosis and pro-apoptotic protein markers decreased (Figure 3). Hence,

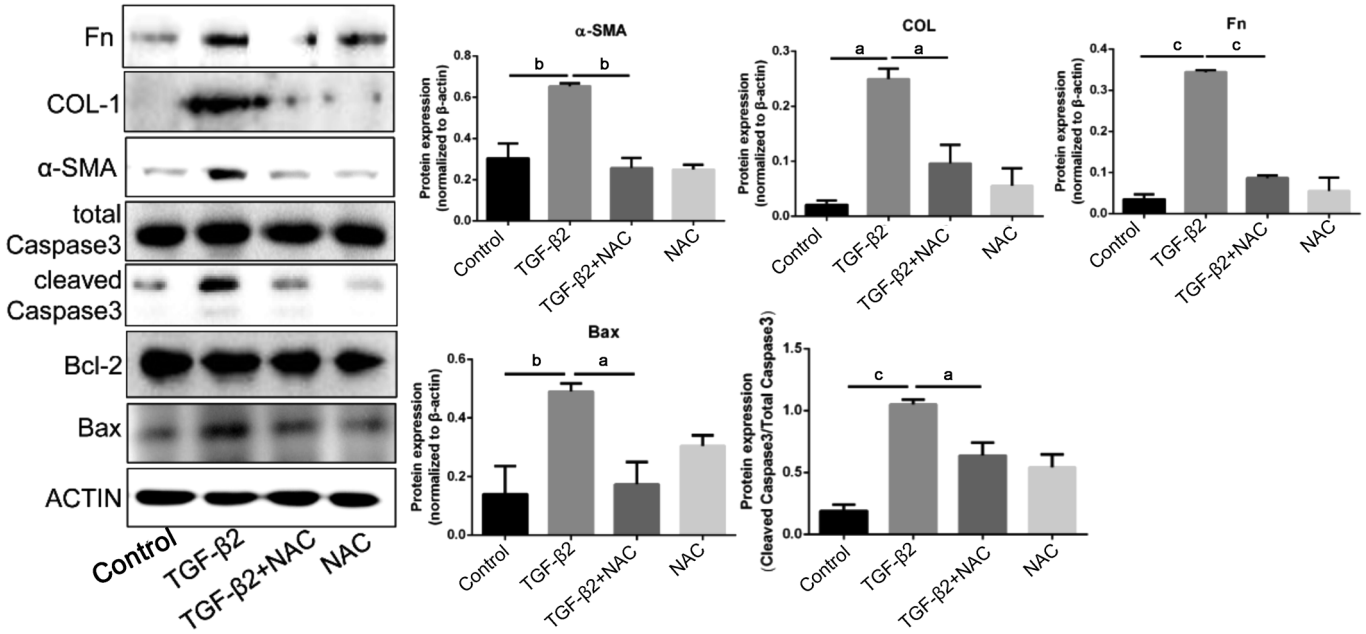

Figure 3 ROS elimination decreases EMT/ECM synthesis and apoptosis-associated protein expression HLECs were pretreated with NAC for 2h; then, 1 μg/L TGF-β2 was added. The cells were incubated for 48h and analyzed by Western blotting. Relative protein expression was calculated using Image J. Data were analyzed using a one-way ANOVA and Tukey's test for multiple comparisons. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.0001$. ROS: Reactive oxygen species; EMT: Epithelial-mesenchymal transition; ECM: Extracellular matrix; HLEC: Human lens epithelial cells; TGF-β2: Transforming growth factor β2; α-SMA: α-smooth muscle actin; Fn: Fibronectin; COL-1: Type 1 collagen.

ROS levels affect HLECs EMT/ECM synthesis and apoptosis, suggesting that ROS act as downstream mediators within PCO pathogenesis by promoting fibrosis and cell apoptosis.

ROS Function in Cell Migration and Invasion Conventionally, PCO is characterized by residual lens epithelial cells with abnormal proliferation, transdifferentiation, migration, and collagen production in the residual capsule. We determined that ROS contributes to EMT/ECM synthesis and apoptosis regulation in HLECs. Therefore, to further clarify the role of ROS in PCO, we assessed whether ROS is involved in HLECs migration and invasion. We used a Transwell assay to assess HLECs invasion capability and found that TGF-β2 effectively promoted HLECs membrane invasion compared to that of the control group HLECs ($P<0.0001$; Figure 4A). However, with NAC treatment, the frequency of HLECs invasion decreased significantly ($P<0.01$; Figure 4B). Similar performance was observed in the wound-healing experiment. In the TGF-β2 group, the wound was observed to be almost recovered after 24h induction compared to that of the control group ($P<0.01$; Figure 4C-4D). Contrastingly, in the TGF-β2+NAC group, a wide gap remained following 24h incubation ($P<0.05$; Figure 4C-4D). NAC alone did not notably change the wound-healing rate (Figure 4C-4D). Overall ROS were determined to mediate TGF-β2-induced HLECs migration and invasion, potentially worsening PCO progression.

Effects of H_2O_2 on HLECs Apoptosis and EMT/ECM Synthesis Thus far, we demonstrated that ROS mediate EMT/ECM synthesis, migration/invasion, and apoptosis in HLECs; however, whether a direct treatment with ROS has the same effects is currently unknown. Therefore, we used H_2O_2 as a ROS source to evaluate its effects on HLECs. H_2O_2 was observed to cause cell apoptosis, especially late-stage apoptosis, in a dose-dependent manner (Figure 5A-5B). Approximately 40% of the HLECs were determined to be in an apoptotic state following 24h of H_2O_2 treatment (Figure 5B). Interestingly, in the western blot analysis of H_2O_2-treated HLECs, only α-SMA protein expression was upregulated in a dose-dependent manner, whereas COL-1 and Fn protein expression decreased in a dose-dependent manner (Figure 5C), without a statistical difference; this suggested that that ROS alone are not able to induce excessive ECM synthesis *in vitro* in HLECs. Taken together, ROS were determined to be responsible for apoptosis and EMT/ECM synthesis; however, ROS alone is not sufficient for ECM synthesis induction.

DISCUSSION

PCO is the most common complication that occurs following cataract surgery, leading to a severe decrease in visual quality[13]. In this study, TGF-β2 was determined to facilitate ROS production in a time-dependent manner. Nonetheless, the ROS scavenger NAC could inhibit EMT, apoptosis, and TGF-β2-stimulated cell migration. However, H_2O_2-simulated ROS could only induce EMT-associated protein expression but did not directly induce the expression of ECM synthesis proteins, consistent with Das *et al*'s study[14]. In conclusion, ROS may

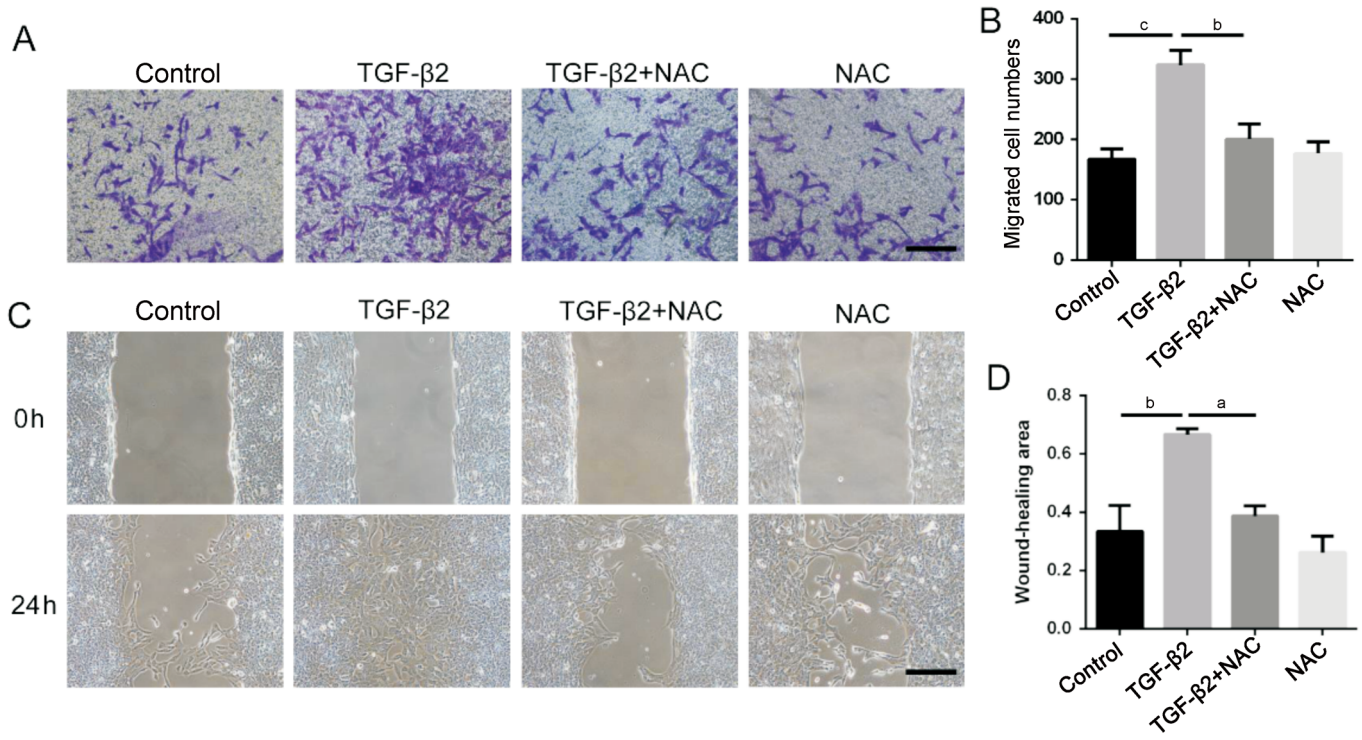

Figure 4 ROS mediate TGF-β2-stimulated cell migration and invasion HLECs were pretreated with NAC for 2h, followed by treatment with 1 μg/L TGF-β2. Cells were incubated for 24h and assessed using Transwell and wound-healing assays. The relative wound-healing area was calculated using Image J. Data were analyzed using a one-way ANOVA and Tukey's test for multiple comparisons. Scale bar: 500 μm, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.0001$. ROS: Reactive oxygen species; HLECs: Human lens epithelial cells; TGF-β2: Transforming growth factor β2; α-SMA: α-smooth muscle actin; Fn: Fibronectin; COL-1: Type 1 collagen.

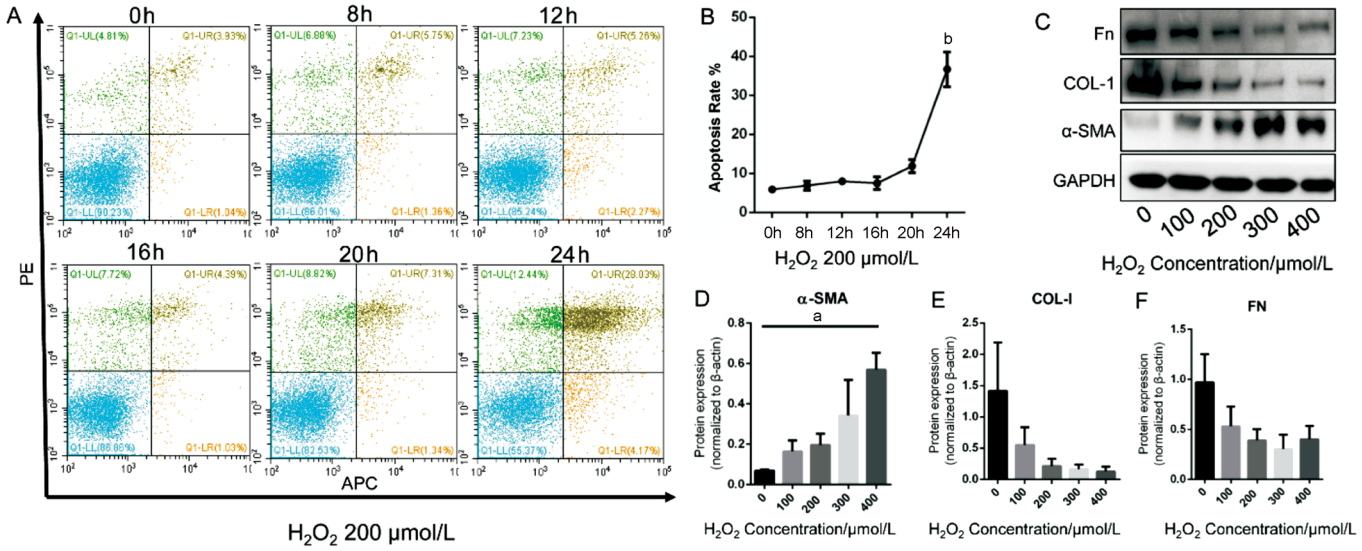

Figure 5 H_2O_2 promoted HLECs apoptosis, α-SMA protein expression but not ECM synthesis A: HLECs were treated with 1 μg/L TGF-β2 across different durations (0, 8, 12, 16, 20, and 24h); HLECs apoptosis was assessed by flow cytometry. B: Apoptosis rate was calculated using a one-way ANOVA and Dunnett's test for multiples of every group, compared to the 0h group; $^{a}P<0.05$, $^{b}P<0.01$. C: Western blot of HLECs treated with H_2O_2 (0, 100, 200, 300, or 400 μmol/L) for 24h. D-F: Relative protein expression was calculated using Image J. Data were analyzed using a one-way ANOVA and Tukey's test for multiple comparisons. ROS: Reactive oxygen species; EMT: Epithelial-mesenchymal transition; ECM: Extracellular matrix; HLECs: Human lens epithelial cells; TGF-β2: Transforming growth factor β2; α-SMA: α-smooth muscle actin; Fn: Fibronectin; COL-1: Type 1 collagen.

act downstream of TGF-β2 and, therefore, promote EMT/ECM synthesis, cell migration, and apoptosis, which coordinate to worsen PCO.

Cell proliferation, migration, transdifferentiation, and ECM synthesis in the lens capsule following cataract surgery are the classical features of PCO[15-20]. Apoptosis in the lens

capsule is also an important characteristic of PCO and anterior subcapsular cataract[21-22]. However, the mechanisms and functions of apoptosis in PCO remain unclear. In this study, we hypothesized that activated-TGF-β2 in the aqueous humor after cataract surgery induces ROS production, thereby contributing to EMT-associated protein expression and ECM synthesis, alongside endogenous apoptosis pathway activation and the upregulation of associated proteins. Thus, ROS-mediated TGF-β2 stimulates multiple signaling pathways, and ROS elimination can reverse these responses.

ROS are highly reactive chemicals formed from O_2 that are frequently derived from the mitochondria of cells, including those in lens epithelial cells[23-24]. ROS are a well-known cause of cellular senescence; consequently, various free radical scavengers are commercially available. Spector and Garner[25] detected that there was approximately 70 μmol/L H_2O_2 in the aqueous humour of cataract patients. Basu *et al*[26] checked endogenous H_2O_2 was in the germinal area of developing lens epithelium, but the concentration was hard to known. As metabolites that diffuses ubiquitously throughout cells, ROS are involved in various cellular processes. At appropriate concentrations, ROS regulate signalling pathways and control specific physiological responses. Nonetheless, shikonin has been determined to inhibit HLECs proliferation by inducing ROS- and caspase-dependent apoptosis. Overall, oxidative stress may activate TGF-β *via* the production of ROS and, consequently, alter the expression of several survival genes, such as lens epithelium-derived growth factor[7]. Wang *et al*[27] recently determined that ROS production is upregulated after mechanical injury, especially at the wound edge. They also established that H_2O_2 concentration as low as 1 μmol/L could significantly induce cell migration and EMT; additionally, the NADPH oxidase inhibitor diphenyleneiodonium could reverse these responses, aligning with the current study. Various concentration of H_2O_2 might exerts different effects on diverse cells[27]. The changes of ROS levels during PCO process are complex, and further research is needed.

Overall, we determined that ROS mediate apoptosis, EMT/ECM synthesis, and cell migration of HLECs in PCO. Additionally, although ROS are important, ROS alone are not sufficient for the direct induction of ECM synthesis. Therefore, our findings reveal the role of ROS in the development of PCO and thereby provide new insights into PCO pathogenesis.

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Authors' contributions: Ma B was responsible for the study design and performing a critical review of the manuscript. Hu CH and Qi TT were responsible for designing and conducting the study and conducting the literature research. Jing RH conducted the data analysis and wrote the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest: Jing RH, None; **Hu CH,** None; **Qi TT,** None; **Ma B,** None.

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