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Oxidative stress–induced temporal activation of ERK1/2 phosphorylates coreceptor of Wnt/β-catenin for myofibroblast formation in human lens epithelial cells

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Purpose: Posterior capsular opacification (PCO) is the most common complication postcataract surgery, and its underlying mechanisms involve epithelial-mesenchymal transition (EMT) of remnant lens epithelial cells (LECs) in response to drastic changes in stimuli in the intraocular environment, such as oxidative stress and growth factors. Wnt/β-catenin signaling is a major pathway mediating oxidative stress–induced EMT in LECs, but its interplay with other transduction pathways remains little known in the development of PCO. ERK1/2 signaling is the downstream component of a phosphorelay pathway in response to extracellular stimuli (e.g., reactive oxygen species), and its activation regulates multiple cellular processes, including proliferation and EMT. Thus, this study aimed to investigate how ERK1/2 signaling and Wnt/β-catenin pathway crosstalk in oxidative stress–induced EMT in LECs.

Methods: Hydrogen peroxide (H_2O_2) at 50 μM treatment for 48 h was used to establish a moderate oxidative stress–induced EMT model in LECs. ERK1/2 signaling was inhibited using MEK1/2 inhibitor U0126 at 20 μM. Western blotting was used to quantify protein expression of various biomarkers of EMT and phosphorylated components in ERK1/2 and Wnt/β-catenin signaling. LEC proliferation was determined using an EdU staining assay and expression of proliferating cellular nuclear antigen (PCNA). Subcellular localization of biomarker proteins was visualized with immunofluorescent staining.

Results: Under the moderate level of H_2O_2-induced EMT in LECs, ERK1/2 signaling was activated, as evidenced by a marked increase in the ratio of phosphorylated ERK1/2 to total ERK1/2 at early (i.e., 5–15 min) and late time points (i.e., 12 h); the canonical Wnt/β-catenin pathway was activated by H_2O_2 at 48 h. LECs exposed to H_2O_2 exhibited hyperproliferation and EMT; however, these were restored by inhibition of ERK1/2 signaling demonstrated by reduced DNA synthesis and PCNA expression for cellular proliferation and altered expression of various EMT protein markers, including E-cadherin, α-SMA, and vimentin. More importantly, inhibition of ERK1/2 signaling reduced β-catenin accumulation in the activated Wnt/β-catenin signaling cascade. Specifically, there was significant downregulation in the phosphorylation level of LRP6 at Ser 1490 and GSK-3β at Ser 9, the key coreceptor of Wnt and regulator of β-catenin, respectively.

Conclusions: ERK1/2 signaling plays a crucial role in the moderate level of oxidative stress–induced EMT in LECs. Pharmacologically blocking ERK1/2 signaling significantly inhibited LEC proliferation and EMT. Mechanistically, ERK1/2 signaling regulated Wnt/β-catenin cascade by phosphorylating Wnt coreceptor LRP6 at Ser 1490 in the plasma membrane. These results shed light on a potential molecular switch of ERK1/2 and Wnt/β-catenin crosstalk underlying the development of PCO.

Posterior capsule opacification (PCO) is the most common complication postcataract surgery and can severely affect the visual quality of patients [1]. The development of PCO is the consequence of a wound healing response of residual lens epithelial cells (LECs) to drastic changes in stimuli in the ocular aqueous environment, such as oxidative stress and growth factors, ultimately leading to LEC hyperproliferation and transdifferentiation from an epithelial morphology to myofibroblast phenotype, known as epithelial-mesenchymal transition (EMT) [1]. In normal eyes, LECs are surrounded in an antioxidant-rich (e.g., glutathione) environment [2]; however, after cataract surgery, abrupt reduction in the antioxidant level accompanied by an elevating oxygen level in the anterior chamber leaves LECs exposed to an environment of redox imbalance, promoting EMT of LECs [3].

Mechanistically, various pathways can cooperate to regulate EMT [4]. Studies of biologic regulatory pathways

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underlying PCO progression have shown that transforming growth factor β (TGFβ) and canonical Wnt/β-catenin signaling in LECs are activated in response to extracellular cues of oxidative stress [5]. TGFβ has been well-recognized as the inductor of EMT by mediating downstream Smads-dependent and -independent signaling pathways [6]. Selective inhibition of NADPH oxidase (NOX), which produces reactive oxygen species (ROS), was shown to block TGFβ-induced EMT of LECs [7]. Furthermore, exposing LECs to a moderate level of oxidant (e.g., H_2O_2) can activate the Wnt/β-catenin signaling–mediated EMT process in the mouse cataract PCO model and primary porcine LECs [5]. However, under oxidative stress conditions, whether TGFβ and Wnt/β-catenin signaling crosstalk triggers EMT in LECs remains to be elucidated.

ERK1/2 signaling is one of the important TGFβ Smads-independent pathways that is implicated in multiple biologic processes, such as survival, cell-cycle progression, proliferation, apoptosis, transcription, differentiation, and EMT [8,9]. Activated ERK1/2 participates in the Ras-Raf-MEK-ERK signal transduction cascade by dual phosphorylation at Tyr and Thr. In LECs, activation of ERK1/2 was shown to cross-interact with TGFβ-Smads signaling, and blocking ERK1/2 signaling with receptor tyrosine kinase (RTK) antagonists or MEK1/2 inhibitor U0126 can effectively inhibit TGFβ-induced EMT in LECs [10-13], suggesting the potential role of ERK1/2 signaling in LECs' EMT.

Under moderate oxidative stress conditions, Wnt/β-catenin is the major pathway mediating EMT in LECs [14]. To activate Wnt/β-catenin signaling, the subsequent phosphorylation of coreceptor LRP5/6 at five repeat PPPS/TP motifs is a key step for signal transduction from membrane to nucleus. ERK1/2 is an important MAPK that phosphorylates the repertoire of substrates to regulate cellular processes, including proliferation and EMT. It is well-known that activation of ERK1/2 specifically phosphorylates substates at Ser/Thr sites directed by proline (Pro-Xxx-Ser/Thr-Pro) [15]. As ERK1/2 can be activated by extracellular stimuli, such as ROS (especially H_2O_2 [8,16]), we hypothesize that ERK1/2 may participate in the canonical Wnt/β-catenin signaling that leads to proliferation and EMT in LECs under moderate oxidative stress conditions. Thus, the aim of this study is to elucidate the potential link between ERK1/2 signaling and Wnt/β-catenin signaling under moderate oxidative stress conditions in LECs.

METHODS

Cell culture and maintenance: This study was approved by the Ethic Committee of Northwestern Polytechnical University. The human transfected lens epithelial cell line HLE-B3 was purchased from the American Type Culture Collection (Rockville, MD). Nineteen short tandem repeat (STR) loci plus the gender-determining locus, amelogenin, were amplified using the commercially available EX20 Kit from AGCU (Wuxi, China). The cell line sample was processed using the ABI Prism® 3500 Genetic Analyzer. Data were analyzed using GeneMapper® ID-X v1.2 software (Applied Biosystems, Suzhou, China). Appropriate positive and negative controls were run and confirmed for each sample submitted. The STR analyses are presented in Appendix 1. Cells were cultured in Eagle's Minimum Essential Medium (Biological Industries, Watertown, MA) containing 10% fetal bovine serum (FBS; Biological Industries) at 37 °C with a 5% CO_2 atmosphere.

Cell treatment: The H_2O_2 solution (3%w/w) was purchased from Sigma-Aldrich (St. Louis, MO). A selective inhibitor of MEK1/2, U0126 was purchased from MCE (Shanghai, China). Five million cells were seeded in a 90 mm cell culture dish and cultured to a confluence of 70%–80%. The cells were starved without FBS for 24 h and then treated with 50 μM of H_2O_2 for 48 h. For the proliferation assay, ERK1/2 signaling was inhibited by 20 μM of U0126 for 2 h before exposure to H_2O_2. For immunoblotting biomarkers of Wnt/β-catenin and EMT, ERK1/2 signaling was inhibited at 20 μM of U0126 for 2 h followed by an additional 6 h cotreatment with H_2O_2.

Western blotting of protein expression: The treated cells were washed with ice-cold PBS (1X; 136 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2-7.4), and total protein was extracted using RIPA lysis buffer. Proteins were detected with western blotting using antibodies for E-cadherin (1:5,000, Proteintech, Wuhan, China), α-SMA (1:1,000, CST, Danvers, MA), vimentin (1:1,000, CST), Snail (1:1,000, CST), phospho-ERK1/2 (1:1,000, CST), ERK1/2 (1:2,000, Proteintech), PCNA (1:5,000, Proteintech), Wnt 10a (1:1,000, Abcam, Cambridge, UK), phospho-LRP6 (Ser1490, 1:1,000, CST), LRP6 (1:1,000, CST), phospho-GSK-3β (Ser9, 1:1,000, CST), GSK-3β (1:1,000, CST), β-catenin (1:5,000, Proteintech), and β-actin (1:1,000, CST). After immunoblotting with primary antibodies, proteins were incubated with horseradish peroxidase-conjugated secondary antibody. Immunoblots were developed using the Omni-ECL™ Chemiluminescence Detection Kit (Yamei, Shanghai, China) and visualized with the Chemiluminescence Imaging System (VILBER FUSION FX6.EDGE). The integrated density of protein bands was

measured with Image J software (J 1.41; National Institutes of Health, Bethesda, MD) after the background was subtracted to calculate the protein expression levels.

Immunofluorescence detection of protein expression and localization: A total of 1.5 × 10^4 cells in 100 µl medium were seeded in the center of a glass dish and cultured for 24 h followed by H_2O_2 treatment. The cells were washed with PBS three times and fixed with 4% paraformaldehyde for 15 min. The cell membrane was permeabilized with 0.2% Triton X-100 for 30 min. Nonspecific sites were blocked with 1% BSA (Solarbio, Beijing, China) for 1 h. Cells were incubated with anti-rabbit phospho-ERK1/2 antibody (1:100, CST, Danvers, MA), and anti-mouse vimentin antibody (1:500, Abcam, Cambridge, UK) overnight at 4 °C followed by washing with PBS containing 0.05% Tween-20 (PBST) three times. Then, p-ERK1/2 was stained with anti-rabbit Alexa Fluor 555-conjugated secondary antibody (1:500, CST), and vimentin was stained with anti-mouse Alexa Fluor 488-conjugated secondary antibody (1:500, CST) in dark conditions for 2 h at room temperature. Residual antibodies were washed away with PBST. Nucleus were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 5 min in dark conditions. Pictures of stained cells were captured using a confocal laser scanning microscope (OLYMPUS, Tokyo, Japan).

EdU staining proliferation assay: Cell proliferation was assessed using an EdU Staining Kit (BeyoClick™ EdU-594, Beyotime, Shanghai, China). A total of 5 × 10^5 cells/well were seeded in a 24-well plate and cultured for 24 h. Cells were starved without FBS for 24 h, and then treated with 50 µM of H_2O_2 for 12 h. ERK1/2 signaling was inhibited by a pretreatment with 20 µM of U0126 for 2 h before H_2O_2 treatment. The treated cells were incubated with 10 µM of EdU for 4 h at 37 °C. After incubation, cells were fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.5% Triton X-100 for 15 min. EdU detection was performed according to the instructions for the kit. The nucleus was stained using Hoechst 33,342. Images of stained cells were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis: The data in this study were analyzed using Prism 8 (version 8.2.1, GraphPad Software, La Jolla, CA). Comparisons of multiple groups were performed using one-way ANOVA, followed by Dunnett's multiple comparisons test. Comparisons between the two groups were performed with the Student *t* test. Data were presented as mean ± standard error of the mean (SEM), and a p value of less than 0.05 was considered statistically significant.

RESULTS

ERK1/2 phosphorylation was induced at early and later time points by moderate levels of H_2O_2: We first established an oxidative stress–induced EMT model by exposing LECs to various concentrations of H_2O_2 for different time periods. It was found that cell viability was not affected by all treated concentrations of H_2O_2 for incubation periods ranging from 16 h to 72 h (Appendix 2). LECs treated with 50 µM of H_2O_2 for 48 h exhibited a significant decrease in E-cadherin and an increase in α-SMA, indicating H_2O_2 promoted the EMT process in LECs under the conditions (Appendix 2). Whether ERK1/2 signaling was involved in H_2O_2-induced EMT in LECs was investigated. First, we examined p-ERK1/2 levels at 5, 10, 15, 30, and 60 min of H_2O_2 treatment. p-ERK1/2 level began to increase after 5 min of H_2O_2 exposure and increased markedly at 10 min ($p<0.001$; Figure 1A). The increase in p-ERK1/2 was further confirmed with immunofluorescent staining. The fluorescence intensity of p-ERK1/2 was stronger in the H_2O_2-treated LECs than in the untreated controls (Figure 1B). To explore the involvement of ERK1/2 signaling in H_2O_2-induced EMT, the p-ERK1/2 level was detected at 1, 2, 4, 8, 12, 24, and 48 h. The level of p-ERK1/2 was reduced at 2 h and later at 24 h and 48 h. However, ERK activation was observed at 4 h and 12 h, as evidenced by the increased level of p-ERK1/2 (Figure 1C). These results suggested that ERK1/2 signaling was amplified at the early and later time points during the moderate H_2O_2-induced EMT process in LECs.

Wnt/β-catenin signaling was activated in H_2O_2-induced EMT: To investigate whether Wnt/β-catenin signaling was involved in H_2O_2-induced EMT of LECs, we first detected the protein expression of EMT biomarkers. It was found that E-cadherin was remarkably downregulated ($p = 0.0094$), whereas α-SMA ($p = 0.0091$), vimentin ($p = 0.0062$), and Snail ($p = 0.0048$) were obviously upregulated after exposure to H_2O_2 (Figure 2A). The data showed that exposing LECs to 50 µM of H_2O_2 for 48 h resulted in significant upregulation of Wnt10a, p-LRP $6^{Ser1490}$, p-GSK-$3\beta^{Ser9}$, and β-catenin ($p = 0.008$ for Wnt10a, $p = 0.03$ for p-LRP$6^{Ser1490}$, $p = 0.016$ for p-GSK-$3\beta^{Ser9}$, $p = 0.01$ for β-catenin; Figure 2B). CLSM images showed that β-catenin (red fluorescence) was translocated from membrane to nucleus (blue fluorescence) in H_2O_2-treated cells, whereas it was mainly expressed on the membrane in the untreated controls (Figure 2C). These results suggested canonical Wnt/β-catenin signaling was activated in the moderate of H_2O_2-induced EMT in LECs.

ERK1/2 signaling promotes LEC proliferation during H_2O_2-induced EMT: LEC hyperproliferation and EMT are the two vital pathological processes contributing to the formation of

PCO. ERK1/2 signaling is an important pathway that participates in proliferation. Whether ERK1/2 signaling is involved in LEC proliferation in response to H_2O_2 was examined. ERK1/2 signaling was inhibited using the selective MEK1/2 inhibitor U0126 at 20 µM. LEC proliferation was evaluated at the DNA replication level based on EdU staining and expression of PCNA, a cofactor of DNA polymerase delta. It has been shown that expression of PCNA was obviously increased in the H_2O_2-treated cells compared to the controls (p = 0.005). Blocking ERK1/2 signaling resulted in partial inhibition of PCNA expression induced by H_2O_2 (Figure 3A). EdU staining further indicated that the percentage of fluorescence intensity of the EdU-positive cells/Hoechst stains cells was 2.7-fold higher in the H_2O_2-treated cells than in the controls (p = 0.03). Blocking ERK1/2 signaling significantly inhibited LEC proliferation compared to the H_2O_2-treated groups (p = 0.01) and the controls (p = 0.04; Figure 3B). These data suggested that ERK1/2 signaling can activate the machinery of DNA replication to promote LEC proliferation during the H_2O_2-induced EMT process in LECs.

Blocking ERK1/2 signaling abolished H_2O_2-induced EMT: To explore whether ERK1/2 signaling was essential for H_2O_2-induced EMT in LECs, ERK1/2 signaling was blocked using U0126. Blocking ERK1/2 signaling had different

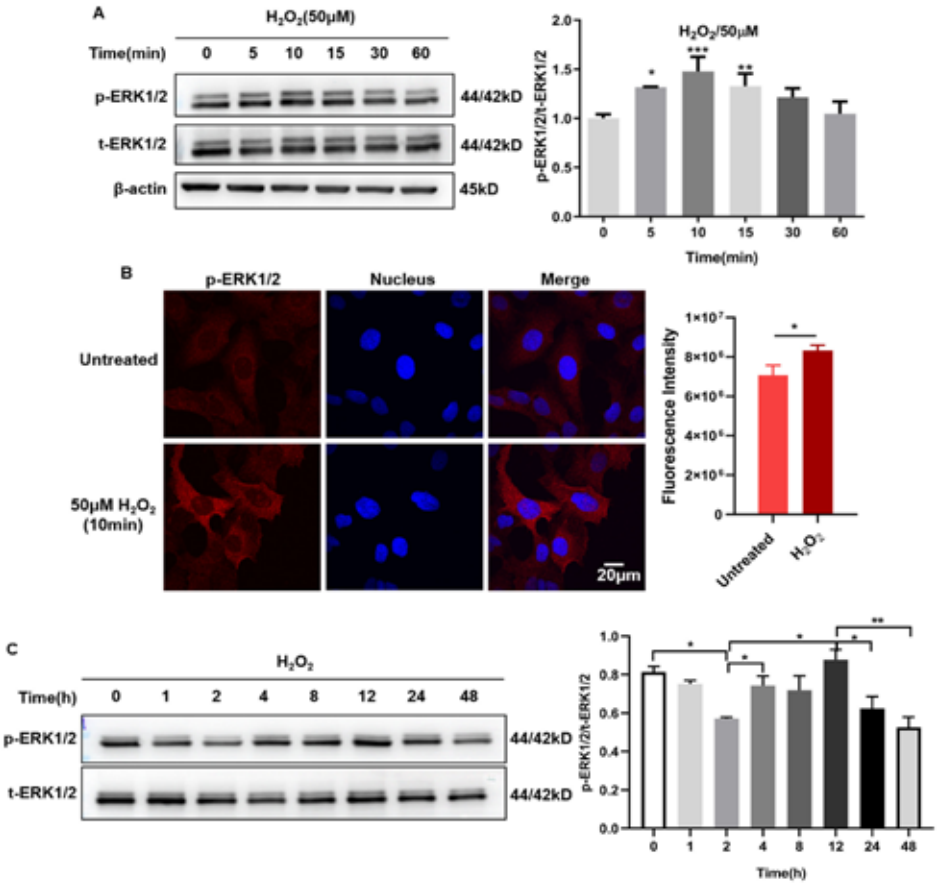

Figure 1. Exposing LECs to moderate H_2O_2 resulted in the upregulation of phosphorylated ERK1/2 at both early and late time points. **A**: Western blotting of p-ERK1/2 and t-ERK/2 in lens epithelial cells (LECs) after treatment with 50 µM of H_2O_2 for 5, 10, 15, 30, and 60 min. Statistical analysis of the p-ERK1/2 to t-ERK1/2 ratio at different time points was performed with one-way ANOVA followed by Dunnett's multiple comparisons test (*p<0.05, **p<0.01, and ***p<0.001). **B**: Immunofluorescent staining of p-ERK1/2 after exposing LECs to 50 µM of H_2O_2 for 10 min. **C**: Western blotting of p-ERK1/2 and t-ERK1/2 after treatment of LECs with 50 µM of H_2O_2 for 1, 2, 4, 8, 12, 24, and 48 h. Statistical analysis of the p-ERK1/2 to t-ERK1/2 ratio was performed with the Student *t* test. The data are shown as mean ± standard error of the mean (SEM) with n = 3.

degrees of inhibitory effects on EMT markers induced by H_2O_2. Upregulation of vimentin and Snail induced by H_2O_2 was completely inhibited by ERK1/2 signaling inactivation (Figure 4A). Downregulation of E-cadherin and upregulation of α-SMA induced by H_2O_2 were also reversed after ERK1/2 signaling inhibition compared to the controls (p>0.05; Figure 4A). CLSM images of vimentin immunofluorescent staining showed that H_2O_2 treatment induced a myofibroblast-like phenotype of LECs, in which green fluorescence–labeled vimentin was prominently distributed in cytoplasm. In contrast, blocking ERK1/2 signaling obviously inhibited the expression of vimentin (Figure 4B). Taken together, these results suggest that ERK1/2 signaling is required for H_2O_2-induced EMT in LECs.

ERK1/2 signaling was involved in LRP6^{Ser1490} phosphorylation in H_2O_2-induced EMT: Because ERK1/2 is the key downstream component of the phosphorelay pathway, and activation of Wnt/β-catenin signaling requires phosphorylation of LRP6 at Ser 1490 within the first PPPS/TP motif and GSK-3β at the Ser 9 site, we first investigated the effect of ERK1/2 signaling on p-GSK-3β^{Ser9} and β-catenin. Western blotting of protein expression showed that blocking ERK1/2 signaling effectively inhibited upregulation of p-GSK-3β^{Ser9}/t-GSK-3β and β-catenin induced by H_2O_2 (Figure 5A). Whether

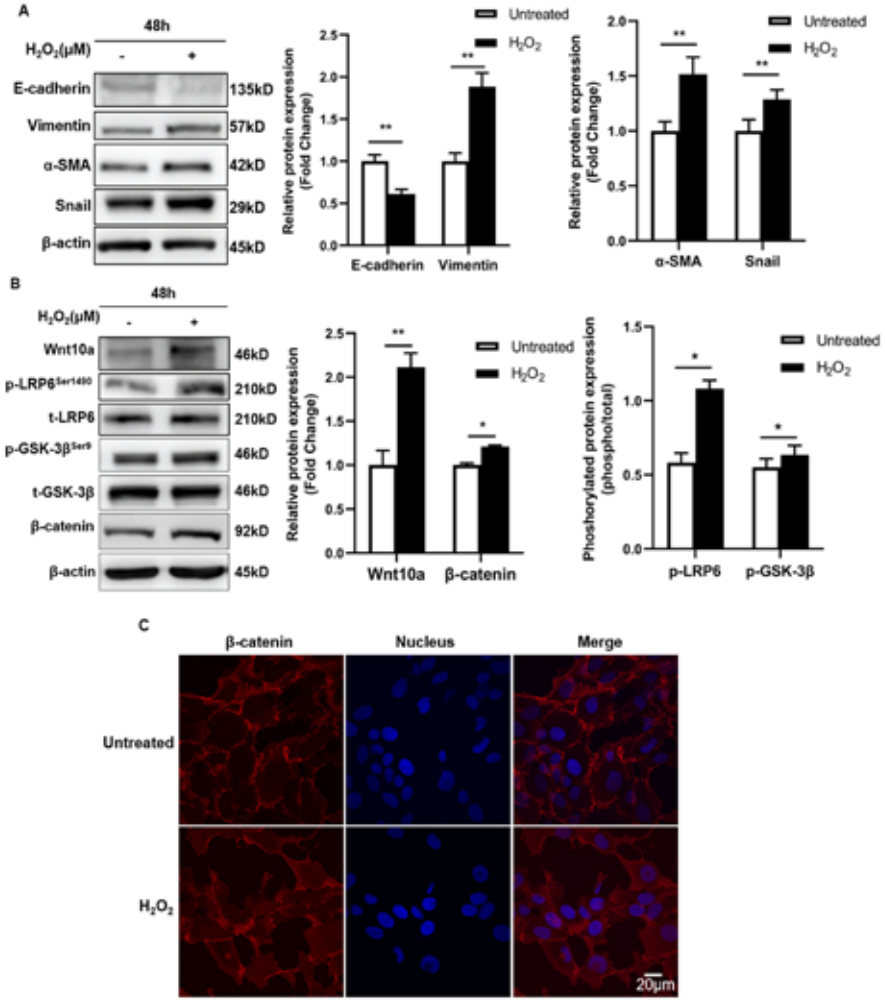

Figure 2. Exposing LECs to 50 μM of H_2O_2 for 48 h activated Wnt/β-catenin signaling and promoted EMT. **A**: Western blotting of E-cadherin, vimentin, α-SMA, and Snail. **B**: Western blotting of Wnt 10a, p-LRP6^{Ser1490}, p-GSK-3β^{ser9}, and β-catenin. **C**: Immunofluorescent images of β-catenin. Statistical analysis was performed with the paired Student *t* test (*p<0.05 and **p<0.01). The data are shown as mean ± standard error of the mean (SEM) with n = 3.

the decline in p-GSK-3β^{Ser9}/t-GSK-3β resulted from inhibition of LRP6 phosphorylation at Ser 1490 by ERK1/2 inactivation was investigated. As expected, LRP6 phosphorylation at Ser 1490 was completely inhibited by ERK1/2 signaling blockage ($p<0.01$; Figure 5C). These results demonstrated that ERK1/2 signaling is linked to Wnt/β-catenin cascade by phosphorylating LRP6 at Ser 1490 in the H_2O_2-induced EMT in LECs.

DISCUSSION

Moderate oxidative stress contributes to the pathogenesis of many chronic diseases, including PCO [7,17,18]. In response to drastic changes in the intraocular microenvironment post-cataract surgery, the residual LECs on the anterior capsule highly proliferate and transdifferentiate to a myofibroblast phenotype. In this study, we found that in moderate oxidative stress–induced LECs' EMT processes, ERK1/2 and Wnt/β-catenin pathways were involved (Figure 6).

It has been shown that ERK1/2 signaling is temporally activated at early and later time points (Figure 1A,C) in response to moderate H_2O_2. The regulation of ERK1/2 signaling by H_2O_2 may be attributed to the extent of activation of kinase (e.g., MEK1/2) and phosphatase (e.g., protein tyrosine phosphatase) by H_2O_2 [19,20]. Activated kinase increases the phosphorylation level, whereas activated phosphatase reduces the phosphorylation level. Zhou et al. [21] reported that MAP kinase phosphatase-1 (MKP-1) is directly activated by H_2O_2 at different time points (i.e., 2 h and 6 h). Xu et al. [22] reported that H_2O_2 increases the expression of MKP-1 at 2 h. These results may explain our observation of the decreased p-ERK1/2/t-ERK1/2 ratio at 2 h and later time points, at 24 h and 48 h.

Activation of ERK1/2 significantly promoted DNA synthesis, suggesting ERK1/2 signaling is an underlying pro-proliferative factor in the development of PCO. Inhibition of ERK1/2 signaling significantly inhibited DNA replication but resulted in only partial downregulation of PCNA expression. It is likely that other pathways (e.g., JNK/SAPK) activated by H_2O_2 were also involved in modulating PCNA expression in LECs [23,24].

PCO occurs as a result of LEC EMT and concomitant deposition of extracellular matrix (ECM), wrinkling of the posterior capsule. LEC EMT is triggered by drastic changes in the capsular bag microenvironment, such as an increase in ROS, cytokines, and growth factors. Wnt family proteins are

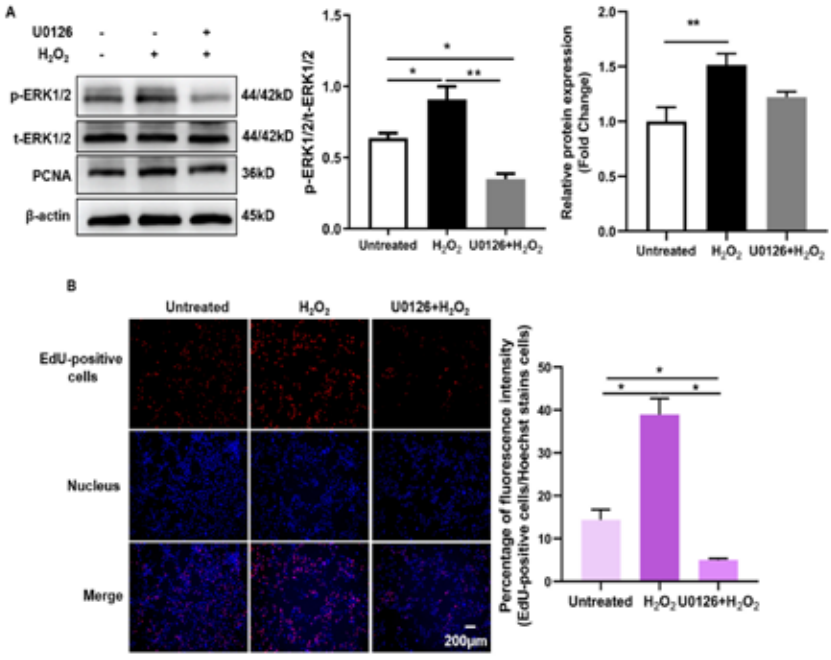

Figure 3. ERK1/2 signaling promoted LEC proliferation in response to moderate H_2O_2 stimulus. **A**: Western blotting of p-ERK1/2, t-ERK1/2, and PCNA. **B**: EdU staining of the lens epithelial cells (LECs). ERK1/2 signaling was inactivated with pretreatment of LECs with 20 μM of U0126 for 2 h before exposure to 50 μM of H_2O_2 for 12 h. Statistical analysis was performed with the paired Student *t* test (*$p<0.05$ and **$p<0.01$). The data are shown as mean ± standard error of the mean (SEM) with n = 3.

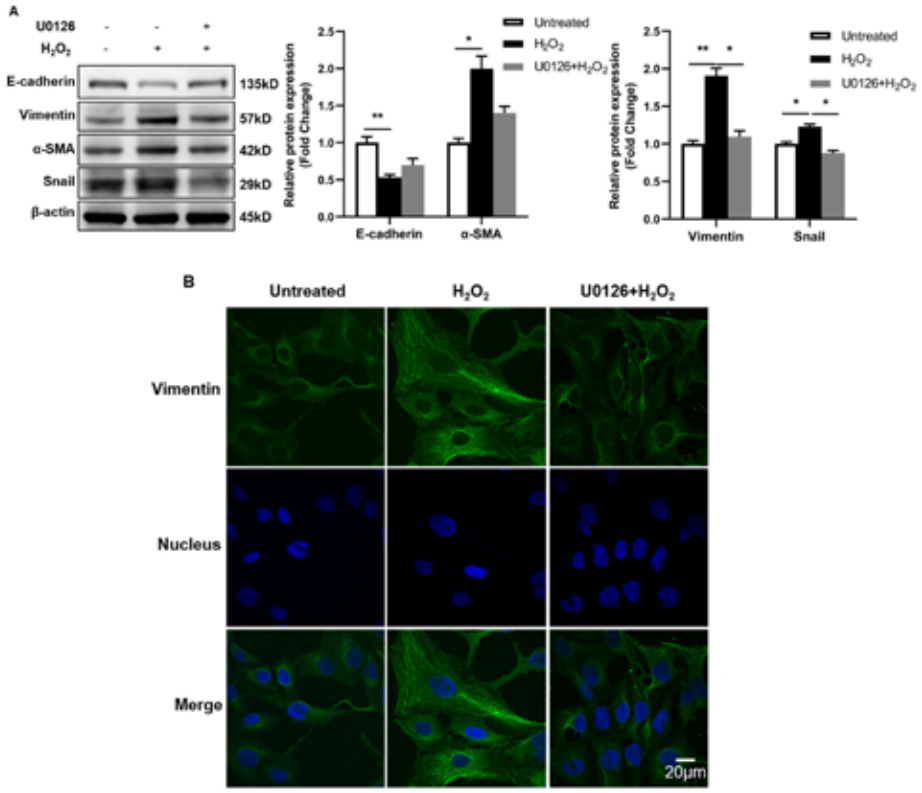

Figure 4. Blocking ERK1/2 signaling prevented moderate H_2O_2-induced EMT in LECs. **A**: Western blotting of epithelial-mesenchymal transition (EMT) biomarkers, including E-cadherin, vimentin, α-SMA, and Snail. **B**: Immunofluorescent image of vimentin. Statistical analysis was performed with the paired Student *t* test using the integrated density of the protein band (*p<0.05 and **p<0.01). The data are shown as mean ± standard error of the mean (SEM) with n = 3.

an important kind of growth factor that plays a role in lens development and fibrotic pathology through participating in the Wnt/β-catenin pathway [25]. Although the Wnt family proteins consist of several Wnt types (e.g., Wnt 3a and Wnt5a) [26], the present data showed that Wnt 10a was remarkably upregulated in response to a moderate level of H_2O_2 stimulus, which was consistent with our previous findings [5]. Wnt 10a upregulation was also found when LECs underwent EMT under the condition of GSH deprivation [27]. These findings indicate that Wnt 10a is sensitive to oxidative stress and can activate Wnt/β-catenin signaling to mediate oxidative stress–induced EMT in LECs.

Aberrant activation of the TGFβ and Wnt/β-catenin pathways is known to promote EMT and EMT-driven fibrosis. Thus, the targeted blockage of signaling pathways that mediate EMT is expected to inhibit EMT-driven fibrotic diseases, including PCO. The present data suggested that blocking ERK1/2 signaling using a small molecule inhibitor U0126 remarkably prevented Wnt/β-catenin signaling and EMT protein marker expression induced by moderate levels of H_2O_2 in LECs (Figure 4 and Figure 5). It has been shown that phosphorylation of GSK-3β at Ser 9 and accumulation of β-catenin were significantly inhibited after ERK1/2 signaling inactivation (Figure 5A,C). Interestingly, LRP6 phosphorylation at Ser 1490 was also completely prevented after ERK1/2 signaling inactivation. The Ser 1490 site was located in the first PPPS/TP motif of LRP6, and its adjacent amino acid sequences revealed that it was a potential substrate that can be phosphorylated by ERK1/2 (Pro-Xxx-Ser/Thr-Pro; Figure 5B) [28,29]. Phosphorylation of LRP6 at Ser 1490 is required for sequential S/T phosphorylation within the other four PPPS/TP motifs and activation of LRP6 function [30]. Although GSK-3β and casein kinase 1γ (CK1γ) are the identified kinases that phosphorylate LRP6 at the five PPPS/TP motifs, other kinases, such as G-protein-coupled receptor kinase (GRK5/6), cyclin-Y-dependent PFTK (PFTAIRE kinase), and MAPKs are also found to be able to phosphorylate LRP6 at the five motifs [30]. It has been reported GSK-3β prefers to phosphorylate substrates that have been prephosphorylated by other kinases (“priming kinases”) [31]. ERK1/2 is one of the

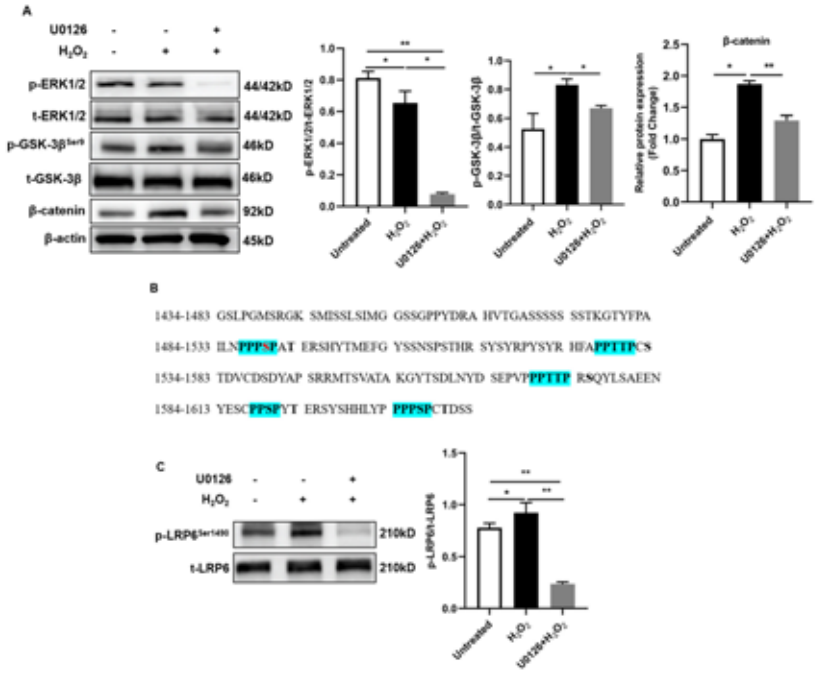

Figure 5. Blocking ERK1/2 signaling prevented Wnt/β-catenin signal transduction in LEC under moderate oxidative stress. **A**: Inactivation of ERK1/2 signaling inhibited p-GSK-3β^{Ser9} and β-catenin. **B**: Intracellular domain (ICD) of LRP6, five PPPS/TP motifs within ICD are highlighted, and Ser 1490 is marked in red. **C**: Inactivation of ERK1/2 signaling inhibited LRP6 phosphorylation at Ser 1490 (*p<0.05 and **p<0.01). Statistical analysis was conducted with the paired Student *t* test using the integrated density of the protein band. The data are shown as mean ± standard error of the mean (SEM) with n = 3.

"priming kinases" that phosphorylates the substrates before being phosphorylated by GSK-3β [32,33]. Because ERK1/2 was activated as early as 10 min, it is likely that ERK1/2 phosphorylates LRP6 at Ser 1490 before GSK-3β in LECs under the condition of moderate H_2O_2 exposure. The present results showed that blocking ERK1/2 signaling led to the complete inhibition of LRP6 phosphorylation at Ser 1490. Therefore, the decline in GSK-3β phosphorylation at Ser 9 likely resulted from the inhibition of LRP6 phosphorylation at Ser 1490 by ERK1/2 signaling inactivation. For Wnt/β-catenin signaling transduction, LRP6 phosphorylation at Ser1490 provides a docking site for the scaffolding protein

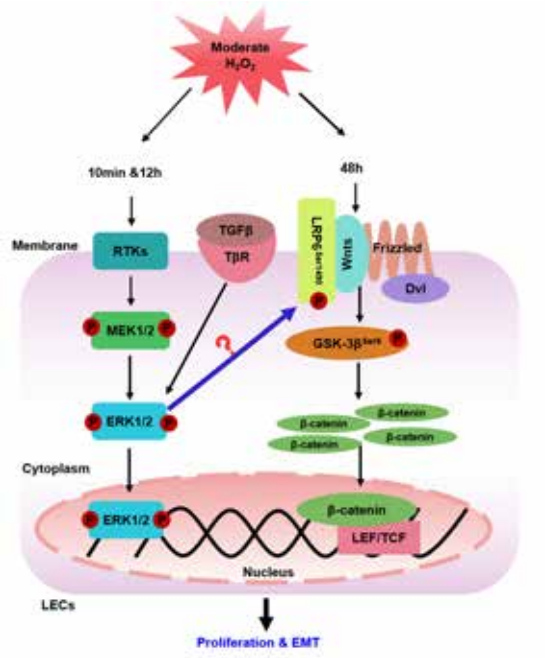

Figure 6. Schematic crosstalk between ERK1/2 and Wnt/β-catenin signaling under moderate levels of H_2O_2 exposure, leading to proliferation and EMT in LEC.

Axin, which leads to the release of GSK-3β from the destruction complex composed by Axin, APC, CK1, and GSK-3β, and followed by GSK-3β phosphorylation at Ser 9 (inactivation) and β-catenin accumulation [34].

In the present study, we elucidated the roles of ERK1/2 in promoting LEC proliferation and Wnt/β-catenin signaling transduction during moderate H_2O_2-induced EMT. Effects of TGFβ in regulating oxidative stress–induced EMT still cannot be ignored owing to the reciprocal relationship between ROS and TGFβ [35]. ROS can stimulate TGFβ expression and activation, which, in turn, promotes ROS generation by decreasing mitochondrial complex IV activity and activating the cytoplasmic NOX system. The interplay between ROS and TGFβ may comprise a key regulatory mechanism underlying LEC EMT and the development of PCO. Importantly, TGFβ can activate ERK1/2 and Wnt/β-catenin signaling through activating TβRs kinase and decreasing Dickkopf-1 expression, respectively [36]. The participation of TGFβ in moderate oxidative stress–induced EMT in LECs, particularly the activation of ERK1/2 interacting with Wnt/β-catenin signaling, is an important aspect to explore further.

Conclusions: In summary, this study demonstrated ERK1/2 signaling plays a crucial role in the moderate oxidative stress–induced EMT process in LECs. Activation of ERK1/2 directly phosphorylates LRP6 at Ser 1490, one of the key Wnt coreceptors. Phosphorylation of LRP6 is required for phosphorylation of GSK-3β at Ser 9, which inhibits β-catenin phosphorylation and promotes β-catenin translocation from cytoplasm to nucleus. The present results revealed a critical regulatory crosstalk between ERK1/2 and Wnt/β-catenin underlying moderate oxidative stress–induced EMT in LECs.

APPENDIX 1. STR ANALYSIS.

To access the data, click or select the words "Appendix 1."

APPENDIX 2. H_2O_2 INDUCES EPITHELIAL-MESENCHYMAL TRANSITION (EMT) IN HLE-B3 CELLS.

To access the data, click or select the words "Appendix 2." A: Cell viability variations after 20, 50, 100 μM of H2O2 treatment for 16, 24, 48 and 72 h, evaluated by MTT assays. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparisons test. B-E: Western blotting of E-cadherin and α-SMA expression. Statistical analysis was performed by paired Student t-test using the integrated density of the protein bands (* $p<0.05$, ** $p<0.01$). The data were shown as mean ± SEM with n=3.

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