

# Protective effect of Glutaredoxin 1 against oxidative stress in lens epithelial cells of age-related nuclear cataracts

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Cataracts, characterized by opacification of the crystal- 90% of cysteine residues and 45% of methionine residues

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**Purpose:** Glutaredoxin 1 (Grx1) is a key antioxidant protein that catalyzes disulfide redox reactions. In this study, we investigated the expression and protective effect of Grx1 against oxidative stress in nuclear cataracts.

**Methods:** Human anterior capsule membrane samples were obtained from the eyes of cataract patients (experimental group) and non-cataractous (control group) donors. The levels of Grx1 protein and mRNA expression were investigated. The human lens epithelial (HLE) cell line SRA 01/04 was transfected with Grx1-containing plasmid or Grx1 small interfering RNA, and cultured under H<sub>2</sub>O<sub>2</sub> treatment, mimicking oxidative stress conditions. Cell counts, clone formation, cell apoptosis, cell cycle, and levels of oxidized glutathione disulfide and cellular reactive oxygen species (ROS) were evaluated and quantified.

**Results:** Protein and mRNA transcript levels of Grx1 were significantly lower in the human anterior capsule membrane of the age-related nuclear (ARN) cataract group than in the control group. Grx1 overexpression protected HLE cells from H<sub>2</sub>O<sub>2</sub>-induced oxidative damage, including alleviating G1 phase arrest, promoting cell proliferation, reducing cell apoptosis, and decreasing intracellular ROS generation. Furthermore, extracellular-signal-regulated kinase (ERK) phosphorylation in the human anterior capsule membrane of ARN patients was higher in the experimental group than in the control group. Grx1 overexpression reduced the levels of oxidized glutathione disulfide and the phosphorylation of ERK. The administration of an ERK phosphorylation inhibitor, PD98059, induced antioxidant effects in Grx1-silenced cells.

**Conclusions:** Grx1 expression is downregulated in the human anterior capsule membrane of ARN patients, accompanied by an increase in ERK phosphorylation. Thus, Grx1 can protect HLE cells against oxidative stress.

line lens, are the leading cause of blindness worldwide [1-3]. Age-related cataracts (ARC) constitute the majority of cataracts, and age is a major risk factor for their development. It is estimated that by 2050, the population aged 60 years or over will reach 2.1 billion, and current estimates indicate that cataracts will afflict more than 20 million people worldwide by then [4]. The social and economic costs of cataracts are staggering, presenting major public health problems globally [5]. Therefore, understanding the mechanism of an ARC is an important step toward the development of strategies to delay its onset and development.

Oxidative stress is considered the main pathogenesis of ARC formation [6], especially in age-related nuclear (ARN) cataracts. Oxidative changes can be detected at the early stages of a nuclear cataract, and progressively increase in severity as the cataract develops [7]. In particular, about

are oxidized in advanced nuclear cataractous lenses [8]. In addition, a significant loss of reduced glutathione (GSH) has also been reported [9]. Such oxidative changes result in the loss of sulfhydryl groups within the lens, a significant increase in proteins with mixed disulfides, the formation of protein-protein disulfide bonds, protein aggregation, and a reduction in crystalline protein solubility. Oxidative damage to essential biomolecules, such as lipids and nucleic acids, has also been observed. For example, the peroxidation of lipids can induce changes in membrane permeability, as well as alter the microviscosity and structural order of the lipid-protein environment [10], resulting in structural changes of the lens [11]. Oxidative DNA damage induces a wide range of DNA strand breaks within lens epithelial cells [12]. With aging, all the above-mentioned oxidative effects accumulate, declining cellular function and causing cell apoptosis, thereby disrupting physiologic lens functions and contributing to the development of ARC [13]. Therefore, maintaining a reducing environment in the lens plays a critical role in lens transparency.

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Glutaredoxin (Grx), also known as thioltransferase, is a key antioxidant protein that carries out glutathione-dependent disulfide redox reactions that catalyze the reduction of protein–thiol mixed disulfides. Grx can maintain thiol–disulfide redox status under reducing conditions, regulate protein dithiol/disulfide balance, repair and regenerate oxidatively damaged proteins, and re-establish a protein’s function and activity. As a dethiolating antioxidant, Grx has been shown to be involved in many processes involved in cellular regulation. For example, Grx plays an important role in the process of apoptosis by regulating the redox state of AKT [14], caspase-3 [15], and p65-NFκB [16]. Grx regulates the cell cycle, DNA replication, and damage repair processes via the p53/p21/p16 signaling axis [17]. Grx can also alleviate oxidative stress-induced cytotoxicity by regulating the Akt-FoxO1, JNK, and/or NF-κB signaling pathways [18-20].

Grx expression and activity have been observed in ocular tissues, such as the iris, ciliary body, cornea, lens, and retina [21]. In retinal glial (Müller) cells, Grx has been shown to resist oxidative stress by regulating NF-κB activity [22] and preventing AKT glutathionylation [23]. Studies have demonstrated that Grx activity is higher in the lens epithelial layer [24] but distributed evenly in the rest of the lens, with little difference between the cortex and the nucleus, and there is a gradual loss of Grx activity in the lens tissues with increasing age [25]. The absence of Grx is known to increase lens susceptibility to oxidative stress [26]. However, the exact mechanism of Grx activity is not well understood. In this study, Grx expression in the human anterior capsule of ARN cataract patients and the effect of Grx on human lens epithelial cells (HLEs) were investigated.

## METHODS

This study adhered to the Declaration of Helsinki, and approval for the use of human anterior capsule membrane samples was obtained from the Ethics Committee of the Eye, Ear, Nose, and Throat (ENT) Hospital of Fudan University, Shanghai, China. Informed consent was obtained from all subjects after an explanation of the study.

*Human anterior capsule membrane samples:* According to the modified version of the lens opacity classification system III (LOCSIII), the type and severity of ARCs were graded and recorded. The types of ARCs were divided into cortical cataracts (C), nuclear cataracts (N), and subcapsular (P) cataracts using a severity grading range from 1 to 6 (the larger the number, the more severe the cataract). Intact human anterior capsule membranes were obtained from the eyes of cataract patients during continuous curvilinear capsulorhexis in surgery, and care was taken to avoid vascular contact,

or damage to the iris or other intraocular structures. Non-cataractous donor eyes were obtained from the Eye Bank of the Eye and ENT Hospital of Fudan University within 24 h of death. The anterior capsule membrane samples from non-cataractous donor eyes were removed and processed in a similar manner to those from cataract eyes, and used as controls. Three anterior capsule membranes of the same type and severity were then homogenized and treated as one sample for subsequent experiments.

*Cell culture and Grx1 overexpression or knockdown transfection:* A human lens epithelial cell line (SRA 01/04), which was authenticated using short tandem repeat (STR, Appendix 1) analysis, was cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco-BRL, Grand Island, NY) with 10% certified fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin, and seeded in 6-well culture dishes at a density of  $2-3 \times 10^4$  cells per well for 12 h, before transfection, in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. DNA fragments encoding human Grx1 were chemically synthesized by Genomeditech Company and inserted into pcDNA3.1 to construct sense plasmids (pcDNA3.1-Grx). Lipofectamine 2000 reagent (Invitrogen) was used to transfect 2 μg of pcDNA3.1-Grx per dish according to the manufacturer’s instructions. Empty vector pcDNA3.1 was used as a negative control. The extent of overexpression was evaluated by western blot analysis.

DNA fragments encoding the Grx1 small interfering RNA (siRNA-Grx, 5'-GCCGCTTGACGTATAGATAC-3') were purchased from Genomeditech Company. The siRNA transfection reagent (13,778,150; Thermo Fisher Scientific) was used to transfect 100 nmol/L of siRNA-Grx per dish according to the manufacturer’s instructions. A scramble (Scr) sequence was used as a negative control. The extent of knockdown was evaluated by western blot analysis.

*Cell proliferation assay:* The Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan) was used to measure the cell proliferation rate according to the manufacturer’s instructions. After 24 h of pcDNA3.1-Grx or siRNA-Grx transfection, SRA 01/04 cells were treated with 200 μM H<sub>2</sub>O<sub>2</sub> solution (Sigma-Aldrich, Shanghai, China) for 3, 6, 12, 18, or 24 h. After H<sub>2</sub>O<sub>2</sub> treatment, the cells were incubated with 10 μL of CCK-8 for 2 h. The optical density (OD) at 450 nm, which was proportional to the number of living cells in the culture, was measured using a microplate reader (Model 550, Bio-Rad, CA).

*Cell clone formation experiment:* After 24 h of pcDNA3.1-Grx or siRNA-Grx transfection, the SRA 01/04 cells were washed with phosphate-buffered saline (PBS) and cultured on coverslips. Seven days later, cells on the coverslips were fixed with 4% paraformaldehyde for 15 min, stained with

0.1% crystal violet for 15 min at room temperature, and photographed with a Nikon inverted microscope.

**Identification of apoptotic cells via flow cytometry:** An annexin V apoptosis kit (556,547, BD) was used to evaluate cell apoptosis according to the manufacturer's protocol. After 24 h of pcDNA3.1-Grx or siRNA-Grx transfection, the SRA 01/04 cells were treated with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> solution for 24 h. After H<sub>2</sub>O<sub>2</sub> treatment, cells were trypsinized and stained with annexin V and propidium iodide (PI), and then analyzed by flow cytometry (FC500; Beckman Coulter, Indianapolis, IN) to differentiate viable, early apoptotic, and late apoptotic cells.

**Cellular reactive oxygen species and oxidized glutathione disulfide detection:** C11-BODIPY<sup>581/591</sup> assay (Invitrogen/Life Technologies) was used to detect cellular reactive oxygen species (ROS) following the manufacturer's instructions. After 24 h of pcDNA3.1-Grx or siRNA-Grx transfection, SRA 01/04 cells were treated with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich), PBS, or 1% extracellular-signal-regulated kinase (ERK) inhibitor (PD98059) for 12 h, and then incubated in 2 mL of medium containing 5  $\mu$ M C11-BODIPY<sup>581/591</sup> for 30 min at 37 °C before detachment by trypsin. Harvested cells were washed twice with PBS, and the cell suspension was then subjected to flow cytometry analysis (FC500; Beckman Coulter) to examine the amount of ROS within the cells. The levels of oxidized glutathione disulfide (GSSG) were determined using the GSH and GSSG Assay Kit (S0053; Beyotime, Shanghai, China) based on the manufacturer's instructions and expressed as nmol/mg protein.

**Cell cycle analysis:** A cell cycle staining kit (Multi Science, China) was used to analyze the cell cycle, following the manufacturer's protocol. After 24 h of pcDNA3.1-Grx or siRNA-Grx transfection, the SRA 01/04 cells were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 12 h. After H<sub>2</sub>O<sub>2</sub> treatment, the cells were harvested and washed with ice-cold PBS, vortexed for 5–10 s with the addition of 1 mL of Reagent A and 10  $\mu$ l of Reagent B, and incubated for 30 min at room temperature. Cell cycle analysis was performed directly using a FACScan Calibur flow cytometer equipped with Modfit 2.0 software (Beckton Dickinson, San Jose, CA, USA).

**Western blot analysis:** SRA 01/04 cells, treated either with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 1% ERK inhibitor PD98059, as well as controls, and human anterior capsule membrane samples were homogenized and lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% sodium pyrophosphate, protease inhibitor cocktail). The total protein concentration in the extracts was determined using the BCA reagent (Pierce). Equal amounts of proteins were separated by 15% SDS-PAGE gel, transferred to PVDF membranes, and incubated with appropriate primary and secondary antibodies, following the standard procedures. The primary antibodies were anti-Grx1 (1:1000; 15,804-1-AP, Proteintech), anti-actin (1:60,000; clone AC-74, Sigma), anti-tubulin (1:100,000; Calbiochem, Gibbstown, NJ), anti-Bcl (1:500; BD Biosciences, San Diego, CA), anti-Bax (1:5000; Cell Signaling Technology, Danvers, MA), and anti-ERK (1:1000; #4376, Cell Signaling Technology), anti-p-ERK (1:1000; #4370, Cell Signaling Technology), and anti-SOD (1:1000; #2770, Cell Signaling Technology). The secondary antibodies were anti-rabbit or anti-mouse (both from Jackson Laboratories, Bar Harbor, ME). As previously reported [27], quantitative analysis of the band intensity of the western blots was performed using Image J software (v1.51, National Institutes of Health, Bethesda, MD) to determine changes in the protein levels.

**RNA isolation, cDNA synthesis, and real-time PCR analysis:** Total RNA from the human anterior capsule membrane samples was extracted using the RNeasy Plus Micro Kit (Qiagen, Duesseldorf, Germany), and 1  $\mu$ g of the extracted RNA was used for cDNA synthesis using the SuperScript III Reverse Transcriptase kit (Invitrogen, Darmstadt, Germany). Quantitative real-time PCR was performed using a StepOne-Plus Real-Time PCR System (Applied Biosystems). The primer sequences used for PCR were shown in Table 1.

**Statistical analysis:** All of the above experiments were repeated three times. The data from the triplicates were recorded for subsequent statistical analysis, all of which were conducted using SPSS Version 25 software (IBM, Armonk, NY). The data were analyzed using independent samples *t*-tests and chi-square tests for continuous and categorical variables, respectively, and a *p*-value <0.05 was considered statistically significant.

TABLE 1. THE PRIMER SEQUENCES USED FOR PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
<i>Grx</i>	CACAGCCACCAACCACTACTA	AGCAGTCCCCACTCTGTTG
<i>SOD</i>	GACTGACTGAAGGCCTGCAT	CCCAAGTCTCCAACATGCCT
<i>actin</i>	CTACAATGAGCTGCGTGTGG	AAGGAAGGCTGGAAGAGTGC

## RESULTS

*Grx and SOD were downregulated in human anterior capsule membranes of ARN cataracts:* By homogenizing three anterior capsule membranes into one sample, nine human anterior capsule membranes from non-cataract donors were obtained and homogenized into three samples as controls. For the N2-N4 nuclear cataract (Figure 1A), nine anterior capsule

membranes were collected and homogenized into three samples at each grade. The profiles of Grx and the antioxidant enzyme superoxide dismutase (SOD) were examined. There was a significant downregulation in Grx gene expression in the ARN cataract, and the protein content (Figure 1B), as well as the mRNA levels (Figure 1C,D), of Grx and SOD in the ARN samples were significantly lower than in the control group.

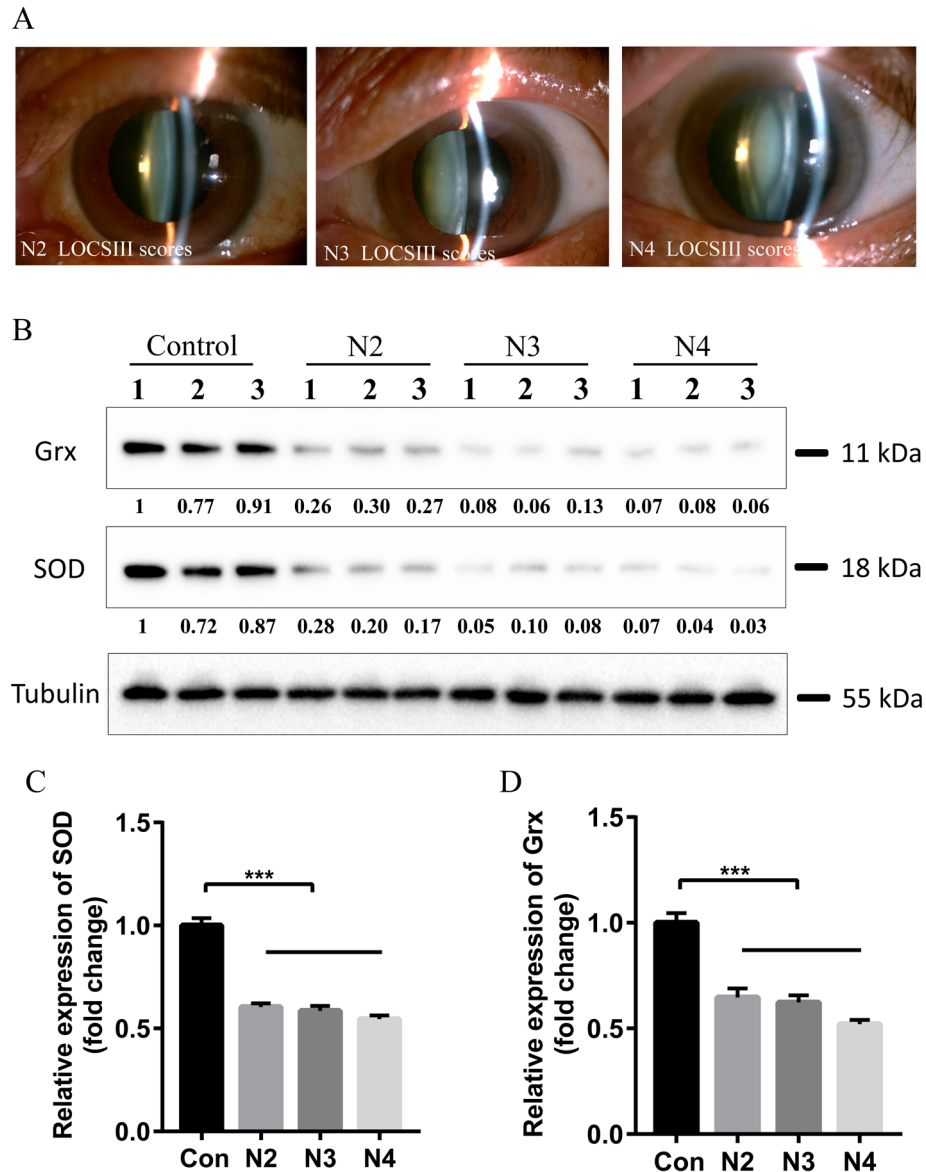


Figure 1. Grx and SOD expression in the human anterior capsule of ARN cataracts. **A:** A slit-lamp photograph of nuclear cataract cases with N2 to N4 LOCSIII scores. **B:** Protein expression levels of Grx and SOD in the lens epithelium of the anterior capsule membrane of age-related nuclear cataract cases and control groups. The quantitative statistical results of the western blot band intensity are labeled under the corresponding western blot band. **C:** mRNA expression of SOD in the lens epithelium of the anterior capsule membrane of the ARN and control groups. **D:** mRNA expression of Grx in the lens epithelium of the anterior capsule membrane of the ARN and control groups. \*\*\*  $p < 0.001$ .

*Overexpression of Grx alleviates cell G1 phase arrest and promotes cell proliferation:* Flow cytometry analysis showed that H<sub>2</sub>O<sub>2</sub> treatment resulted in cell cycle arrest in the G1 phase. After Grx treatment, the percentage of cells in the G1 phase decreased significantly, while the percentage of cells in the S and G2 phases increased significantly (Figure 2A,C). When Grx was silenced, the percentage of cells in the G1 phase increased significantly, while the percentage of cells in the G2 phase decreased significantly (Figure 2B,D). Overexpression of Grx reduced the level of GSSG in cells in an oxidative environment, while knocking down Grx significantly increased the expression of GSSG (Figure 3B). A cell growth curve and clone formation assay were used to detect changes in cell proliferation under various conditions. Upon

extension of H<sub>2</sub>O<sub>2</sub> treatment duration, the cell proliferation rate gradually decreased, demonstrating that H<sub>2</sub>O<sub>2</sub> induces oxidative stress and inhibits cell proliferation. This inhibition was alleviated by the overexpression of Grx, and enhanced by silencing Grx (Figure 3C). A clone formation assay revealed that overexpression of Grx increased colony formation (Figure 3D), while Grx silencing decreased this behavior (Figure 3E). These results suggest that Grx promotes the proliferation of human lens epithelial cells.

*Enforced expression of Grx reduces H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis:* Cells treated with H<sub>2</sub>O<sub>2</sub> demonstrated a significant increase in cell apoptosis (Figure 4B,E). Under oxidative stress conditions induced by H<sub>2</sub>O<sub>2</sub>, the proportion of apoptotic

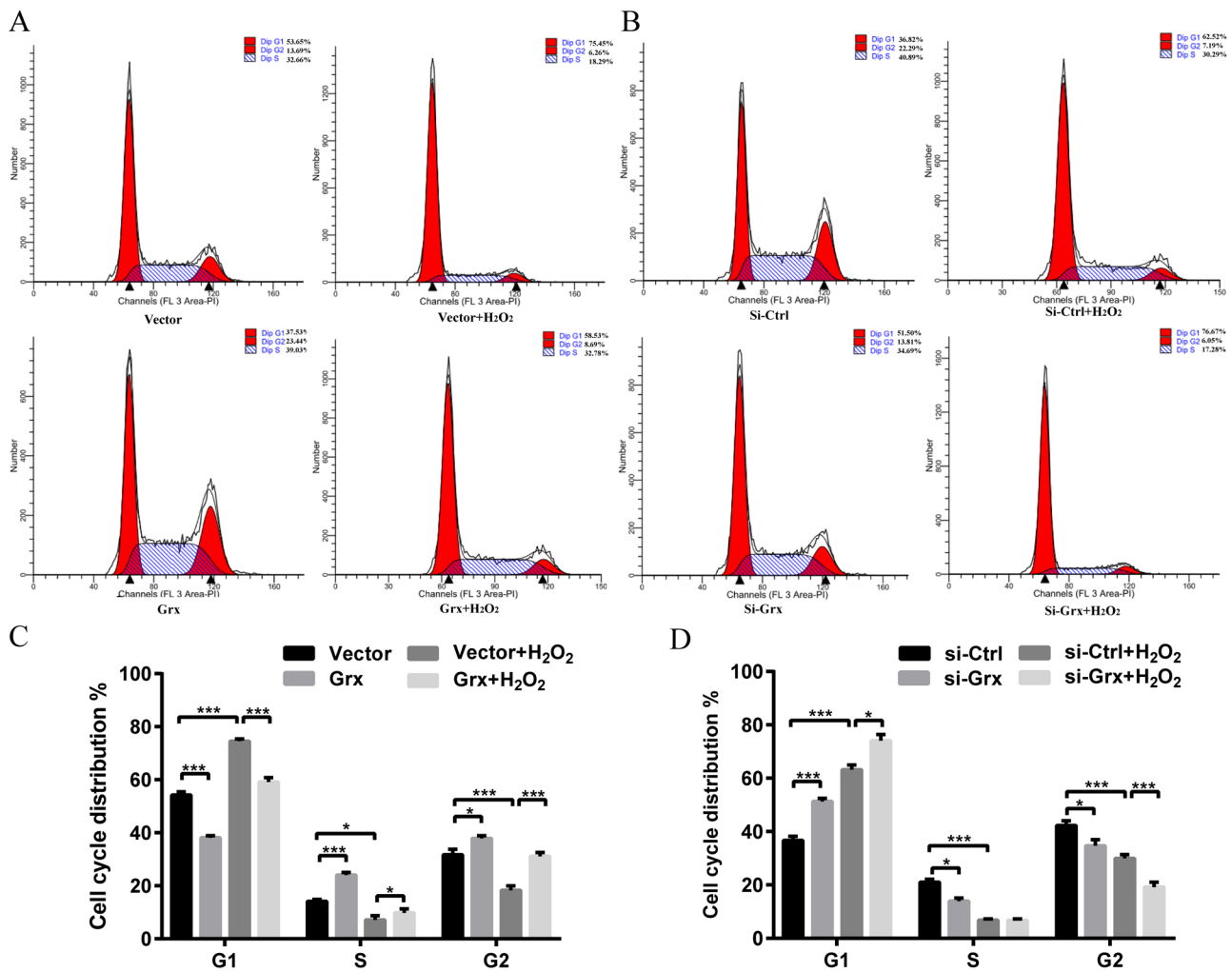


Figure 2. Effects of Grx on the cell cycle. **A:** DNA content profile denoting the cell cycle distribution of HLE cells following treatment with or without 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx overexpression. **B:** DNA content profile denoting the cell cycle distribution of HLE cells following treatment with or without 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx knockdown. **C:** The percentage of HLE cells in the G1/G2/S phase following treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx overexpression. **D:** The percentage of HLE cells in the G1/G2/S phase following treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx knockdown. \*\*\*  $p < 0.001$  and \*  $p < 0.05$ .

cells in the Grx overexpression group was significantly lower than that in the control group (15.14% versus 26.08%, Figure 4A). Grx reduced cell apoptosis by approximately 50%. The proportion of apoptotic cells in the Grx-silenced group was significantly higher than that in the control group (31.51% versus 10.61%, Figure 4D), and Grx silencing resulted in an approximately threefold increase in apoptosis. Interestingly,

higher levels of anti-apoptotic protein Bcl-2, lower levels of pro-apoptotic protein Bax, and decreased cleaved caspase-3 activation were observed in the Grx-overexpression group (Figure 4C), while the corresponding protein expression patterns in the Grx-silenced group were found to be the opposite (Figure 4F).

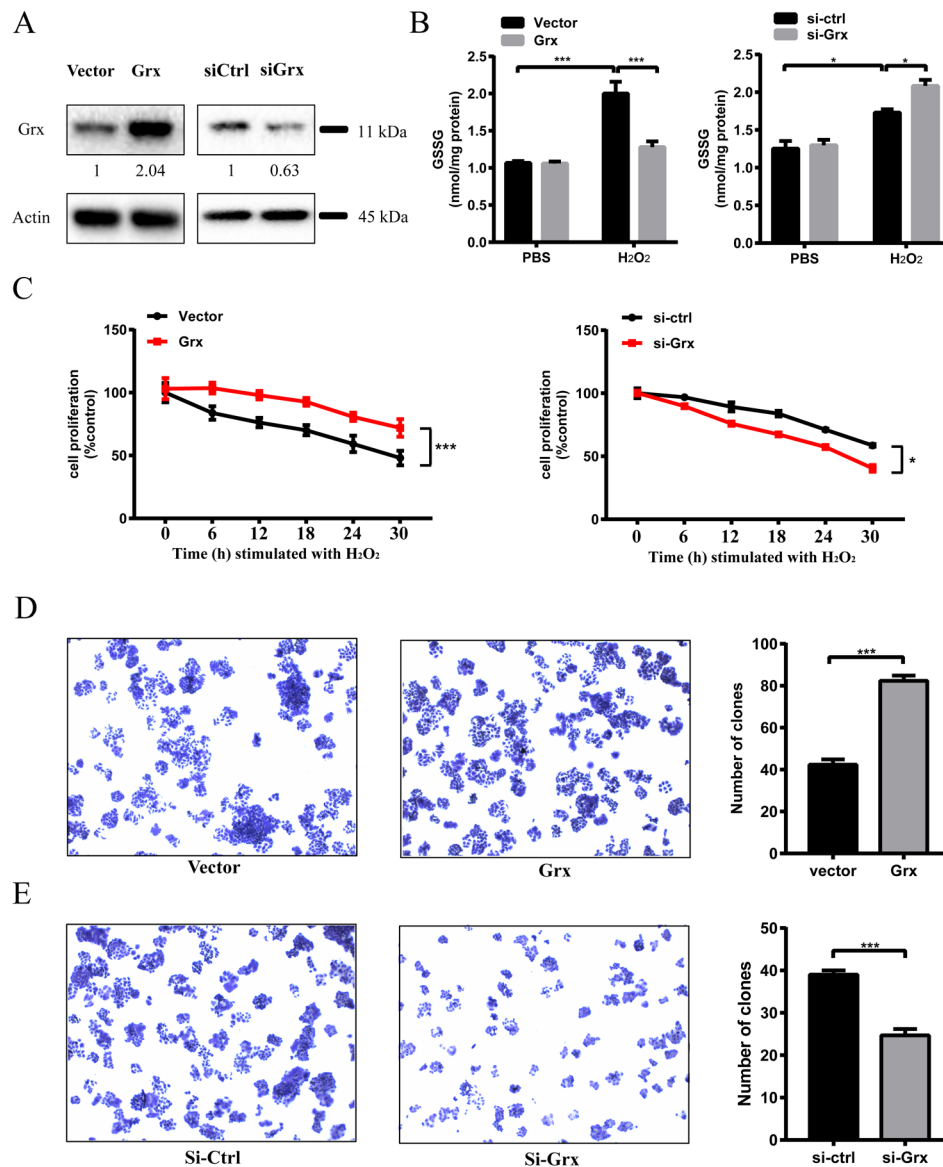


Figure 3. Effect of Grx on cell proliferation. **A**: Detection of Grx expression in Grx overexpression and knockdown transfections. The quantitative statistical results of the western blot band intensity are labeled under the corresponding western blot band. **B**: Levels of oxidized glutathione disulfide (GSSG) in HLE cells after treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h in the Grx overexpression or knockdown groups. **C**: Time course of HLE cell growth following treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for various durations in the Grx overexpression or knockdown group; **D**: Photograph and number of formatted clones of HLE cells following treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h in the Grx overexpression group. **E**: Photograph and number of formatted clones of HLE cells following treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h in the Grx knockdown group. The cell number was measured using an automated cell counter. \*\*\*  $p < 0.001$  and \*  $p < 0.05$ .

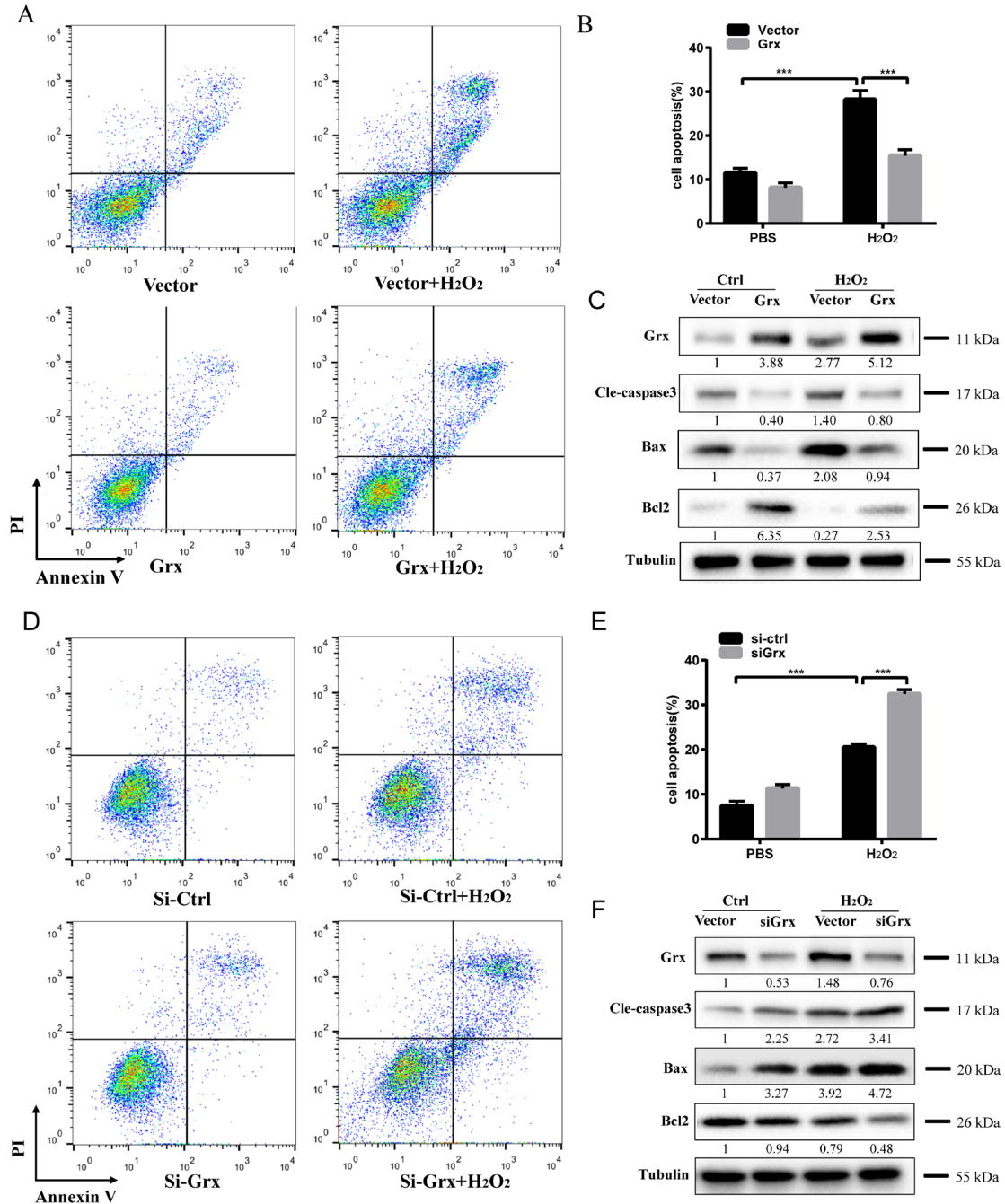


Figure 4. Effect of Grx on cell apoptosis. **A, D:** Flow cytometry analysis of HLE cells following treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx overexpression (**A**) and knockdown (**D**). Early/primary apoptotic cells (Annexin V<sup>+</sup>/PI<sup>-</sup>), late/secondary apoptotic cells (Annexin V<sup>+</sup>/PI<sup>+</sup>), and necrotic cells (Annexin V<sup>-</sup>/PI<sup>+</sup>) were distinguished. **B, E:** Percentage of apoptotic cells following treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx overexpression (**B**) and knockdown (**E**) in HLE cells. **C, F:** Western blot analysis of Bax/BCL2/Cle-caspase3 expression in HLE cells following treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx overexpression (**C**) and knockdown (**F**). The quantitative statistical results of the western blot band intensity are labeled under the corresponding western blot band. \*\*\*  $p < 0.001$ .

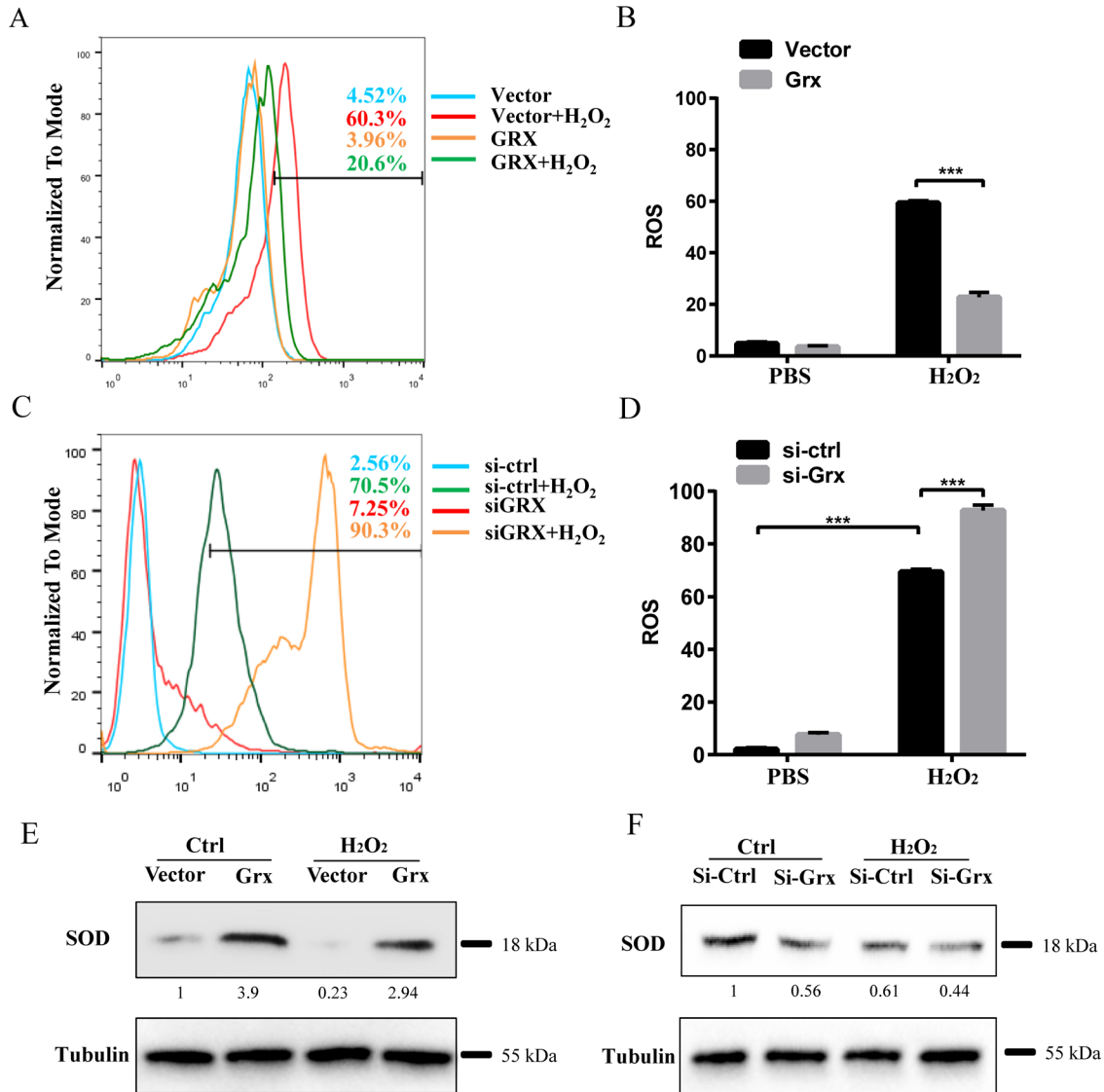


Figure 5. Effect of Grx on intracellular ROS generation. **A, C**: Histograms of intracellular ROS levels in HLE cells following treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx overexpression (**A**) and knockdown (**C**). **B, D**: Bar graph analyses of the median fluorescence intensities of HLE cells following treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx overexpression (**B**) and knockdown (**D**). **E, F**: Western blot analysis of SOD expression in HLE cells following treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx overexpression (**E**) and knockdown (**F**). The quantitative statistical results of the western blot band intensity are labeled under the western blot band. \*\*\*  $p < 0.001$ .

*Grx decreased H<sub>2</sub>O<sub>2</sub>-induced intracellular ROS generation:* Cells treated with H<sub>2</sub>O<sub>2</sub> experienced a significant increase in intracellular ROS levels (Figure 5A–D). Overexpression of Grx significantly reduced intracellular ROS levels (Figure 5A,B), while Grx silencing significantly aggravated the generation of intracellular ROS (Figure 5C,D). The expression of SOD is an indication of the antioxidant defense capabilities of cells. Grx overexpression was accompanied by an increase in SOD expression, while Grx silencing reduced SOD levels

(Figure 5E,F), indicating that the presence of Grx enhances oxidative defense capabilities.

*Grx reduced ERK phosphorylation:* Representative markers of the MAPK and NF- $\kappa$ B signaling pathways, namely p38 and p56, respectively, as well as MEK/ERK protein expression, were observed under oxidative stress conditions in the presence of Grx overexpression. The overexpression of Grx significantly reduced the phosphorylation of ERK but had no effect on the expression of p-p38, p-p56, and p-mek (Figure 6A). Grx silencing promoted the phosphorylation of ERK



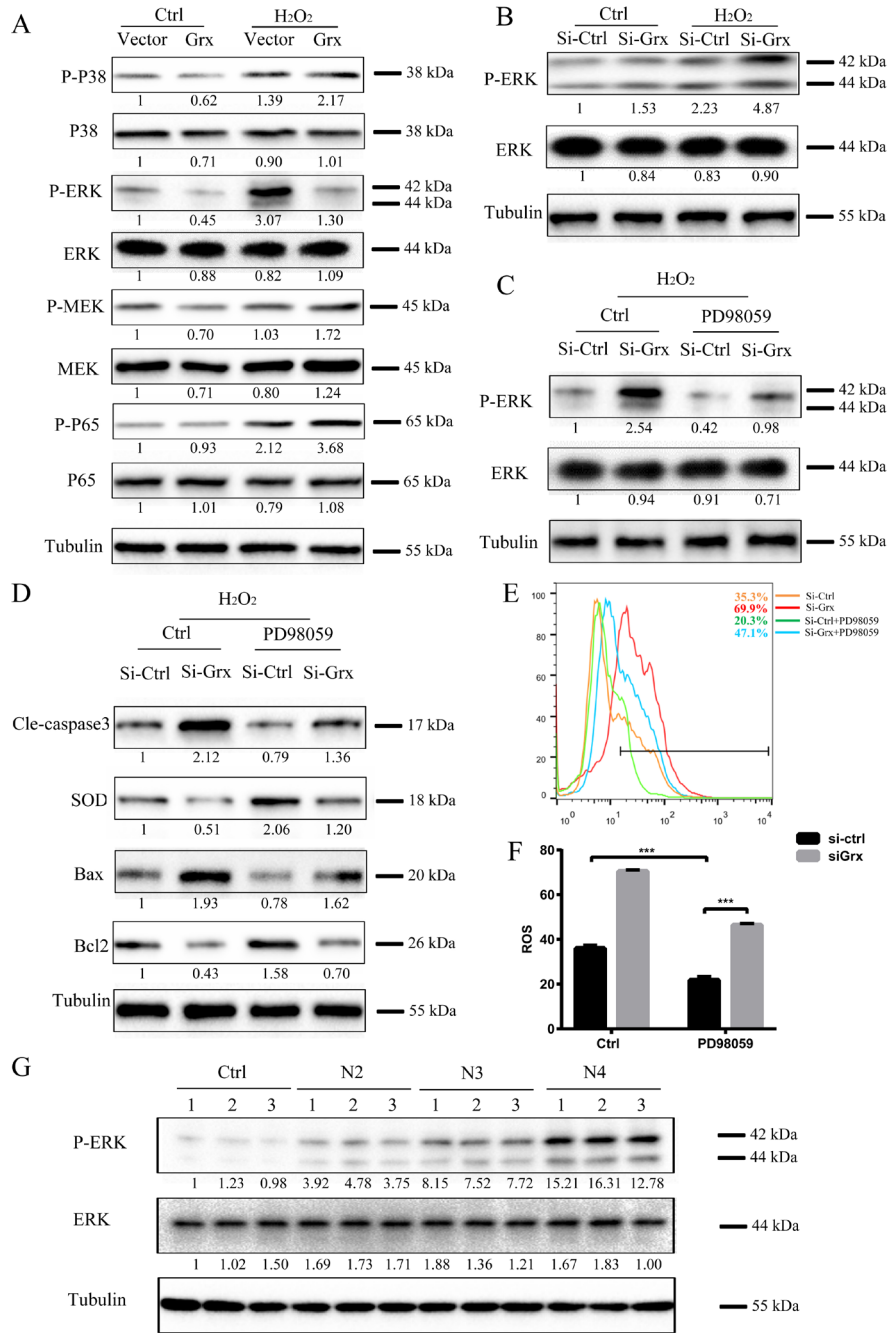


Figure 6. Grx1 substantially inhibits ERK activation. **A**: Western blot analysis of phosphorylated AKT, ERK1/2, and p53 in HLE cells following treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx overexpression. **B**: Western blot analysis of phosphorylated ERK in HLE cells following treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx knockdown. **C**, **D**: Western blot analysis of phosphorylated ERK, BAX, cle-caspase3, BCL2, and SOD in HLE cells pre-treated with 1% PD98059 for 1 h, followed by treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after overexpression transfection. **E**: Histogram of intracellular ROS levels in HLE cells pre-treated with 1% PD98059 for 1 h, followed by treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx overexpression transfection. **F**: Bar graph analyses of the median fluorescence intensities of HLE cells pre-treated with 1% PD98059 for 1 h, followed by treatment with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h after Grx overexpression. **G**: Western blot analysis of phosphorylated ERK in the lens epithelium of the anterior capsule membrane of nuclear cataract patient samples, with N2 to N4 LOCSIII scores, and control groups. The quantitative statistical results of the western blot band intensity are labeled under the corresponding western blot band. \*\*\*  $p < 0.001$ .

(Figure 6B). The phosphorylation of ERK in the anterior capsule membrane of ARN cataract patients was further investigated, and the results showed that the p-ERK protein content of ARN cataract patients was higher than that of the control group (Figure 6G).

The ERK phosphorylation inhibitor PD98059 was then used to inhibit ERK activation in Grx-silenced cells (Figure 6C). Although Grx silencing was accompanied by a decrease in SOD expression, administration of PD98059 was found to induce a decrease in the expression of pro-apoptotic proteins Bax and cleaved-caspase3, increase the level of anti-apoptotic protein Bcl-2 (Figure 6D), and decrease ROS production (Figure 6E,F).

## DISCUSSION

In recent years, the antioxidant effect of Grx has been widely explored in various diseases, such as osteoarthritis [28], allergic airway disease [29], and hepatic lipid disease [30]. In addition, many studies have explored the function of Grx in the lens; for example, the gradual loss of its activity with increasing age [25], the increased susceptibility to oxidative stress in its absence [26], and its ability to increase the duration of lens exposure to UVR by 1.3 times [31]. However, the protective effects of Grx in HLE cells have not yet been fully elucidated. The current study demonstrated the protective role of Grx1 on HLE cells under oxidative stress, and the results showed that Grx can reduce intracellular ROS generation, decrease cell apoptosis, and promote cell proliferation, thus reducing the sensitivity of HLE cells to oxidative damage. Although previous studies have shown that Grx protects the cells from oxidative damage by regulating the Akt, NF- $\kappa$ B, and JNK signaling pathways, we found that Grx can reduce phosphorylation of ERK under oxidative stress in HLE cells, and that the deficiency of Grx1 was accompanied by an increase in ERK phosphorylation in the human anterior capsule of ARN cataract patients.

Previous studies have implicated alterations in Grx expression in a variety of diseases involving oxidative stress. For example, Grx expression is low in Werner and atypical Werner syndrome fibroblasts, which are characterized by oxidative stress-induced DNA damage [32]. The level of Grx is significantly decreased in alveolar macrophages of stage IV chronic obstructive pulmonary disease patients in comparison with healthy non-smokers [33]. Diabetic patients have higher levels of serum Grx than healthy control subjects [34], and Grx protein levels are significantly increased in the cerebrospinal fluid of early-stage Alzheimer's disease patients [35].

In this study, we demonstrated for the first time that the expression of Grx is decreased in the human anterior capsule of ARN cataract patients in comparison with non-cataractous individuals. In addition, the decline in Grx expression in the human anterior capsule of ARN cataract patients does not occur gradually as the severity of cataracts progresses but is significant in the early stages of ARN cataracts (N2 LOCSIII scores). The lens is composed of a capsule formed by a single layer of epithelial cells, and the lens body is formed by lens fiber cells differentiated from epithelial cells. Epithelial cells undergo the elimination of cellular organelles (including nuclei), gradually migrate to the inside of the lens, and differentiate into specialized elongated fiber cells [36,37]. This differentiation will continue with the growth of the lens and will be maintained throughout life; this helps establish the distinctive and unique transparent architecture of the lens. Hence, altered Grx1 expression in the epithelium might reduce the levels of glutathione protein mixed disulfides in subsequently differentiated lens fiber cells located in the interior lens, which might contribute to nuclear cataract formation.

Previous studies have shown that Grx regulates DNA replication [17] and that Grx silencing impairs cell cycle progression at the G(2)/M phase [38]. In this study, cell cycle analysis further confirmed the involvement of Grx in HLE cell cycle regulation, as Grx significantly increased the percentage of cells in the S and G2 phases and decreased the percentage of cells in the G1 phase under oxidative stress. Previous studies have shown that Grx is the hydrogen donor for mammalian S-phase ribonucleotide reductase (RNR), which catalyzes the rate-limiting step in deoxyribonucleotide synthesis and, thus, is essential for DNA replication and repair [39]. In addition, the presence of Grx promotes DNA replication under hypoxic conditions, mitochondrial DNA synthesis, and DNA repair outside of the S-phase [40]. This may, in part, explain the proliferative effect of Grx observed in the present study.

The antioxidant capacity of Grx in HLE cells was also confirmed in this study. To elucidate the role of Grx in lens epithelial cells in response to oxidative stress, HLE cells were cultured with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> to mimic oxidative stress conditions. Upon treating cells with H<sub>2</sub>O<sub>2</sub>, Grx1 overexpression reduced cell apoptosis by approximately 50%, while its silencing resulted in an approximately threefold increase in apoptotic cells. In addition, Grx negatively modulated pro-apoptosis factor Bax, positively modulated anti-apoptosis factor Bcl2, and inhibited caspase-3 cleavage. Furthermore, Grx expression was accompanied by an increase in the expression of the antioxidant enzyme SOD. This indicates

that Grx exhibits oxidative stress defense capabilities and plays a central role in the regulation of oxidative stress-induced apoptosis.

Previous studies have highlighted various anti-oxidative pathways regulated by Grx, such as the activation of Akt [14], the inhibition of JNK activation [18], the glutathionylation of NF- $\kappa$ B [41], and the inhibition of Fas cell surface death receptor (Fas) [42]. This study found that Grx can reduce the phosphorylation of ERK. The ERK pathway plays an essential role in controlling cell proliferation and differentiation. It has been reported to be activated by UV radiation, oxidants, high glucose levels, cellular shock, and cytokines [43,44]. A recent study by Gong et al. [45] implicated a key role of the ERK pathway in cataract formation. In the current study, we found that Grx expression was decreased in the human anterior capsule membrane of ARN patients, and this was accompanied by an increase in ERK phosphorylation. At the same time, Grx could reduce phosphorylation of ERK and intracellular ROS levels in HLE cells, while revealing no regulation of the MAPK, NF- $\kappa$ B, or MER pathways.

In conclusion, this study demonstrated that a deficiency in Grx expression was accompanied by an increase in ERK phosphorylation in the human anterior capsule membrane, and also illustrated the protective effects of Grx on human lens epithelial cells. However, this study has a few limitations. Although intracellular experiments confirmed the protective effect of Grx, the functions of Grx in cultured SRA01/04 cells were not likely to completely mirror the functions of Grx in the intact untransformed lens. Hence, further experiments using primary lens cells or cultured lenses are needed to confirm the protective function of Grx1 in untransformed lenses.

## APPENDIX 1. STR ANALYSIS.

To access the data, click or select the words “[Appendix 1.](#)”

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