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Senescence marker protein30 protects lens epithelial cells against oxidative damage by restoring mitochondrial function

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ABSTRACT

Etiology and pathogenesis of age-related cataract is not entirely clear till now. Senescence marker protein 30 (SMP30) is a newly discovered anti-aging factor, which plays an important role in preventing apoptosis and reducing oxidative stress damage. Mitochondria are located at the intersection of key cellular pathways, such as energy substrate metabolism, reactive oxygen species (ROS) production and apoptosis. Oxidative stress induced by 4-hydroxynonenal (4-HNE) is closely related to neurodegenerative diseases and aging. Our study focused on the effect of SMP30 on mitochondrial homeostasis of human lens epithelial cells (HLECs) induced by 4-HNE. Western blots and qPCR were used to compare the expression of SMP30 protein in the residual lens epithelial cells in the lens capsule of age-related cataract (ARC) patients and the donated transparent lens capsule. On this basis, SMP30 overexpression plasmid and SMP30 shRNA interference plasmid were introduced to explore the effect of SMP30 on the biological behavior in HLECs under the condition of oxidative stress induced by 4-HNE through immunohistochemistry, ROS evaluation, metabolic spectrum analysis and JC-1 fluorescence measurement. Given that Nuclear Factor erythroid 2-Related Factor 2 (Nrf2)/Kelch Like ECH Associated Protein 1 (KEAP1) signaling pathway is the most important antioxidant stress pathway, we further analyzed the regulatory effect of SMP30 by WB to explore its molecular mechanism. Our study indicated that SMP30 may inhibit ROS accumulation, restore mitochondrial function, activate Nrf2/Keap1 signaling pathway, therefore protecting lens epithelial cells from oxidative stress-induced cell damage.

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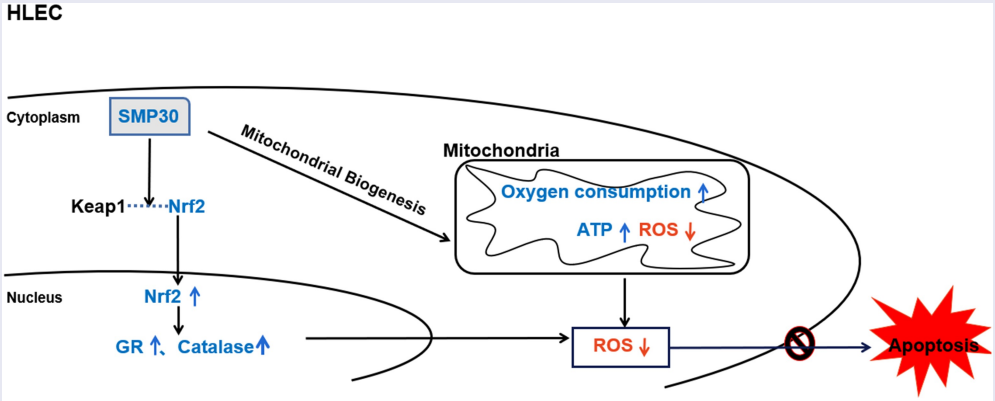

SMP30 protects lens epithelial cells against 4-HNE induced oxidative stress.

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1. Introduction

Cataract is the most common blinding eye disease in the world. To date, only the surgical removal of cloudy lens is the effective treatment method. The pathogenesis of cataract is an extremely complicated subject, and the etiology and pathogenesis of age-related cataract (ARC) are not completely clear. Recent studies have shown that the occurrence of cataract is a multifactorial process, and oxidative damage is considered an important factor in the development of ARC [1–3]. In our previous research, we found that senescence marker protein 30 (SMP30) protein alleviates the apoptosis of human lens epithelial cells exposed to UV irradiation by regulating the expression of apoptosis-related proteins, both UV irradiation and 4-HNE can induce oxidative stress in lens epithelial cells [4].

SMP-30 is a newly discovered anti-aging factor and has become a focus of research in recent years. SMP30 plays an important role in preventing apoptosis and reducing oxidative stress damage [5]. The expression of SMP30 decreased with age and increased under oxidative stress [6]. Oxidative damage produces the metabolite 4-HNE, which is associated with inflammatory pathologies related to oxidative stress, such as neurodegenerative diseases and aging [7]. Increased amounts of 4-HNE may be linked to cataract [8].

As the main producer of reactive oxygen species (ROS) in hypoxic cells, mitochondria determine the fate of cells under hypoxic conditions [9]. Mitochondrion lies at the intersection of critical cellular pathways, such as energy substrate metabolism, ROS generation, and apoptosis [10]. Mitochondrial dysfunction can cause and exacerbate diseases. Oxidative stress is an important factor in cataract formation, and lens epithelial cells induced by oxidative stress are closely related to the formation of ARC [11]. Mitochondria induce oxidative stress, ROS production, and imbalance of the redox state of lens, leading to the formation of human cataract [12]. Mitochondrial homeostasis is the basis of normal cell function. Advanced glycation products can induce oxidative stress and then lead to mitochondrial oxidative damage, which will finally cause cell apoptosis [13]. Therefore, the occurrence of cataract is related to the damage of lens epithelial cells, mitochondrial function, and oxidative stress level. Protecting mitochondrial function and inhibiting apoptosis are essential to the inhibition of ARC progression.

To clarify the role of SMP30 in the pathogenesis of cataract and provide potential biological targets for cataract prevention and treatment, we speculate that SMP30 may protect lens epithelial cells and inhibit cataract formation by inhibiting oxidative stress damage and restoring mitochondrial function.

2. Method

2.1 Donor sample examination

2.1.1 Human sample

The study protocol and informed consent forms were obtained from all the participants, and the tenets of the Declaration of Helsinki were followed. A total of 20 cataract patients (20 eyes) aged 50–75 years who underwent phacoemulsification in Tianjin Medical University Eye Hospital were recruited for this study. Ethics approval was obtained from the Institutional Review Board/Ethics Committee of Tianjin Medical University Eye hospital (2021KY(L)-17, Tianjin, China). A 5–6 mm capsule membrane in the center of the anterior capsule of the lens was removed through curvilinear capsulorhexis in cataract surgery [14]. Transparent lens anterior capsule membranes were obtained from donor eyes (seven cases,14 eyes) for penetrating keratoplasty of the same age group. We examined all the donor eyes with a hand-held slit lamp to ensure that all the lenses were transparent.

2.1.2 Immunohistochemistry

Two groups were plated with paraformaldehyde-fixed capsules, and the expression of SMP-30 in HLECs was detected with streptavidin-peroxidase (SP) immunohistochemistry (SP kit, Solarbio) [15]. Antigen retrieval conditions were 0.01 mol/L sodium citrate buffer at 95°C for 15 min. Rabbit anti-human SMP30 polyclonal antibody (1:150; ab233007, Abcam) was added and incubated at 4°C overnight. Relevant secondary antibodies and other reagents were added in accordance with the SP kit instructions, developed with DAB,

counterstained with hematoxylin, and sealed with neutral gum. PBS solution (P1022, Solarbio) replaced the primary antibody as a negative control. By using an image analyzer, the number of positive cells and the gray value of the positive reaction intensity were measured for semi-quantitative analysis. The gray value is an indicator of the degree of light transmission of a translucent medium and can be divided into 0–256 levels; the strength of the positive response and expression increase with decreasing level.

2.2 In vitro experiments procedure

2.2.1 Cell culture and treatment

A human lens epithelial cell line was provided by Yang Chunbo (Tianjin Medical University, Eye Institute). The cells were grown in Eagle's minimum essential medium containing 10% fetal bovine serum. For transient transfection, plasmids were transfected into cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol [16]. We used 4-hydroxynonenal (4-HNE, Sigma) at a working concentration of 10 μM as a stressor to induce oxidative stress, incubated the cells for 18 h, and collected them for further use [17].

2.2.2 Construction of pcDNA-3.1-SMP30 plasmid

First, an SMP30 fragment was amplified through PCR: homo cDNA and SMP30-FF/SMP30-RR were used as the template and primers, respectively. PCR amplification was performed for the production of an SMP30 fragment [4].

SMP30-FF:
AGCGCTACCGGACTCAGATCTCGAGATGTCTTCCATTAAGATTGA

SMP30-RR:
CCGTCGACTGCAGAATTCGAAGCTTTCCCGCATAGGAGTAG

Digestion reaction: The target fragment SMP30 and the vector pRFP-N1 were digested;

Ligation reaction: The target fragment SMP30 was ligated with the vector pRFP-N1;

Transformation experiment: Approximately 10 μL of recombinant solution was added to 100 μL of DH5a competent cells, which were in turn placed in an ice bath for 30 min, subjected to heat shock at 42°C for 1 min, immediately returned to ice, and left to stand for 2 min. Then, 300 μL of LB was added at 37°C for 30 min of resuscitation. Plating was performed. The cells were placed in a biochemical medium and incubated overnight at 37°C. Monoclonal colonies were collected and expanded for plasmid extraction and sequencing identification. Lipofectamine was used according to the manufacturer's specifications (Invitrogen) for transient transfection.

2.2.3 SMP30 shRNA interference plasmid

The interference sequence was designed as follows:
shRNA sequence:

(+)
GatccGAACAAATCCCAGATGGAATGTGTATTCAAGAGATACACATTCCATCTGGGATTTGTTCTTTTTTc;

(-)
aattgAAAAAAGAACAAATCCCAGATGGAATGTGTATCTCTTGAATACACATTCCATCTGGGATTTGTTCg

The Phb-u6 plasmid was selected as the template, and the upstream and downstream primers of the SMP30 interference plasmid were amplified through PCR [4]. Finally, a hairpin-like structure with a length of about 374 bp was obtained, which contained the U6 promoter and SMP30 target gene. After the enzyme digestion reaction, ligation reaction, transformation experiment, and sequence identification, the recombinant plasmid was finally obtained. Lipofectamine was used according to the manufacturer's specifications (Invitrogen) for transient transfection.

2.2.4 JC-1 fluorescence measurement of the mitochondrial membrane potential

The mitochondrial membrane potential (#KTA4001, Abbkine) was detected using JC-1 fluorescence mitochondrial imaging [18]. The hUSLFs were incubated with JC-1 solution for 20 min at 37°C. The cells were then washed twice with JC-1 buffer, and a medium was added to each well. Images were taken using a fluorescence microscope (Olympus, Tokyo, Japan). The ratio of red to green fluorescence

represented the mitochondrial membrane potential.

2.2.5 Metabolic profile analysis

Analysis of metabolic spectrum by Seahorse XFe96 Flux Analyzer (Agilent, Santa Clara, CA, USA) by simultaneously quantifying oxygen consumption rate (OCR) [19]. The cells were separately transfected with PSF plasmid with a lipofectamine 2000 transfection reagent (Invitrogen, USA). We sequentially added specific compounds related to electron respiration chain, including oligomycin, FCCP, and RAA prepared in the cartridge to track the reaction of a mitochondrion.

2.2.6 H&E staining

The effect of different expression levels of SMP30 on cell morphology was observed through H&E staining (ab245880, Abcam): 3.0×10^4 HLECs were mounted on a 24-well cell culture plate (Stemcell.38017, Coster) [20]. When the cell confluence was about 80%, the transfection was carried out according to the experimental group. After transfection for 24 h, the cells of each group were exposed to 4-HNE (Sigma) for 24 h. After 48 h of incubation, the coverslips were removed and stained with conventional hematoxylin and observed under a fluorescence microscope.

2.2.7 ROS assay

We performed 2,7-dichlorodihydrofluorescein (DCF) diacetate (H2DCFDA) assays (Sigma) to determine intracellular ROS production [21]. HLECs (1.5×10^4) were inoculated into 96-well cell plates with 200 µL culture medium. After the cells reached about 70% confluence, transfection treatment was carried out according to the experimental groups. After 24 h of transfection, the cells were treated with 4-HNE for 24 h and incubated for 30 min with 25 µM DCFH-DA. A cell-permeable fluorogenic probe was used to detect ROS. After the cells were washed with PBS twice, the intracellular accumulation of ROS was determined according to the relative fluorescence intensity at an emission/excitation wavelength of 535/485 nm.

2.1.8 Total ROS assessment by flow cytometry

We measured cell total ROS with a Total ROS Detection Kit 520 nm (88–5930-7488-5930-7, Thermo Fisher, USA). The cell groups were suspended separately in a cell-staining buffer. A single cell suspension was prepared through filtration. According to a previously described method [12], a 5 µM working solution was prepared. Approximately 1.0 mL of a 5 µM reagent was used as a cell loading solution in which cells were incubated for 10 min at 37°C without light exposure. The cells were then gently washed three times with warm PBS. A flow cytometer (Becton, Dickinson and Company, USA) was used in analyzing cells and acquiring data.

2.2.9 Live and dead assay

The HLECs were inoculated in 96-well culture plates, and 3.0×10^4 cells were seeded for the control and experimental groups. After the confluence of the cells grew to about 80%, the cells were transfected. After 24 h, the cells were exposed to 4-HNE for 24 h and then cultured in the complete medium for 24 h. Live and dead staining agents (ab115347, Abcam) were prepared: 2 µL of ethidium homodimer-1 (EthD-1) and 0.5 µL of calcein Am were added to 1 mL of PBS solution, and then 50 µL of live or dead dye solution was added to each hole. The cells were incubated at room temperature for 30 min [22]. The kit provides a ready-to-use reagent that facilitates distinguishing between dead and living cells. The living cells would be marked with green fluorescence, and dead cells would be marked as red cells. The original dye solution was discarded to terminate the staining, and 50 µL PBS solution was added to each hole. Images were obtained under a fluorescence microscope (Olympus, Japan).

2.2.10 Western blot

Cells were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate). Protein lysate (30 µg) was separated on a 10% SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane (Millipore, USA) [23]. The primary antibodies used were anti-Nrf2 (1:1000; Bioss, China), anti-GR (1:1000; Boster, China), anti-catalase (1:1000; Boster, China), and anti Keap1 (1:1000; Boster, China). Anti-GAPDH antibody (1:1000; Cell Signaling Technology, Beverly, MA, USA) was used as a loading control. After washing, the membrane

was incubated with a horseradish peroxidase-conjugated secondary antibody (1:3000, GE Healthcare, Little Chalfont, UK) for 1 h at room temperature. Protein bands were visualized with ECL (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) on an X-ray film, which was quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.3 Statistical analysis

SPSS 19.0 statistical software was used in statistical analysis. One-way analysis of variance (ANOVA) combined with Dunnett's statistical method was adopted. The data were first tested for homogeneity of variance, and then Dunnett was used in making a pairwise comparison between the experimental and control groups. Two independent samples t-test was used to compare the population characteristics of the participants. A P value of <0.05 was considered statistically significant, and the data obtained were graphed with GraphPad Prism software.

3. Result

3.1. Comparison of SMP30 expression between lens epithelial cells isolated from ARC's and transparent lens

To determine the association between SMP30 expression and cataract, we compared the expression of SMP30 in lens epithelial cells isolated from ARC lens and transparent lens. The population characteristics of the participants in this study are shown in Table 1. SMP30 levels in lens epithelial cells isolated from transparent lens (TLECs) and ARC (CLECs) were measured with immunohistochemistry, Western blot, and qPCR. As shown in Figure 1(b–c), SMP30 in the epithelial cells was mainly expressed in the cytoplasm, and the color was brown. The gray values of SMP30 expressed in TLEC and CLECs were 71.36 ± 10.02 and 186.03 ± 16.54, respectively, which indicated that the expression of SMP30 in CLECs was significantly lower than that in TLEC (P = 0.042). The Western blot and qPCR data were consistent (t = 11.04, 41.72; P = 0.0004; P < 0.0001), as shown in Figure 1 (d–f), SMP30 level in the CLECs was significantly lower than that in the TLECs. These data indicated that SMP30 levels are closely related to cataract.

Table 1. Study population characteristics of the participants in this study (mean ± SD).

Characteristic	ARC	Transparent lens	t	P
Number of cases	20	7		
Number of eyes	20	14		
Age, y	67.0 ± 7.04	63.0 ± 6.38		1.3
0.20				2
Gender, M:F	7:13	5:2		
Left eye, %	35	50		

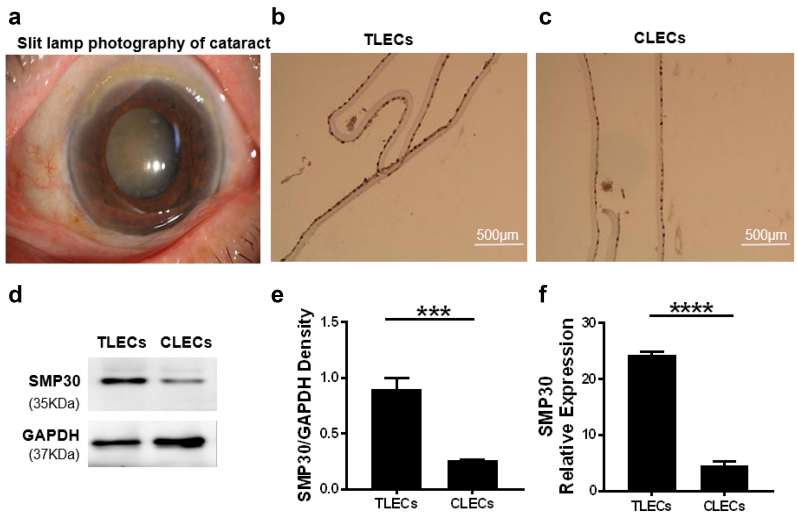

Figure 1. Comparison of SMP30 expression between ARC and clear lens epithelial cells.

(A) Slit lamp photography of cataract. (B) SMP30 staining in transparent lens epithelial cells (TLECs) by immunohistochemistry. (C) SMP30 staining in cataract lens epithelial cells (CLECs) by immunohistochemistry. (D) SMP30 protein level checked by Western blot. (E) SMP30 density analysis. (F) SMP30 mRNA level measured by qPCR.

3.2. Effects of SMP30 on mitochondrial membrane potential of HLECs treated with 4-HNE

To explore the relationship between SMP30 and early apoptosis of lens epithelial cells, we analyzed the effect of SMP30 on mitochondrial membrane potential of HLECs treated with 4-HNE. A mitochondrial membrane potential detection kit (JC-1) was used to detect the early apoptosis of cells according to the principle of mitochondrial membrane potential change (Figure 2). The result showed that the early apoptosis rates (%) were 0.97 ± 0.02 (N), 5.32 ± 0.02 (N + 4-HNE), 6.08 ± 0.04 (4-HNE + pcDNA3.1), 1.81 ± 0.05 (4-HNE + SMP30), 5.29 ± 0.05 (4-HNE + Scramble), and 13.51 ± 0.06 (4-HNE + shSNP30). The difference between the groups was statistically significant ($F = 8689$, $P < 0.0001$). The percentage in the SMP30 group was lower than that in the N group treated with 4-HNE ($F = 8689$, $P < 0.0001$), whereas the early apoptosis (%) in the shSMP30 group was higher than that in the SMP30 group ($F = 8689$, $P < 0.0001$), indicating that 4-HNE can affect the mitochondrial membrane potential of HLECs and then induce cell apoptosis, and SMP30 can obviously inhibit the early apoptosis of HLECs.

(A) Mitochondrial membrane potential detection kit (JC-1) is used to detect early apoptosis of Normal group and N + 4-HNE group cells. (B) 4-HNE + pcDNA3.1 group and 4-HNE + SMP30 group, (C) 4-HNE + Scramble group and 4-HNE + shSMP30 group according to the principle of mitochondrial membrane potential change. (D) Qualitative analysis of the percentage of apoptosis cells in each group. *P < 0.0001.

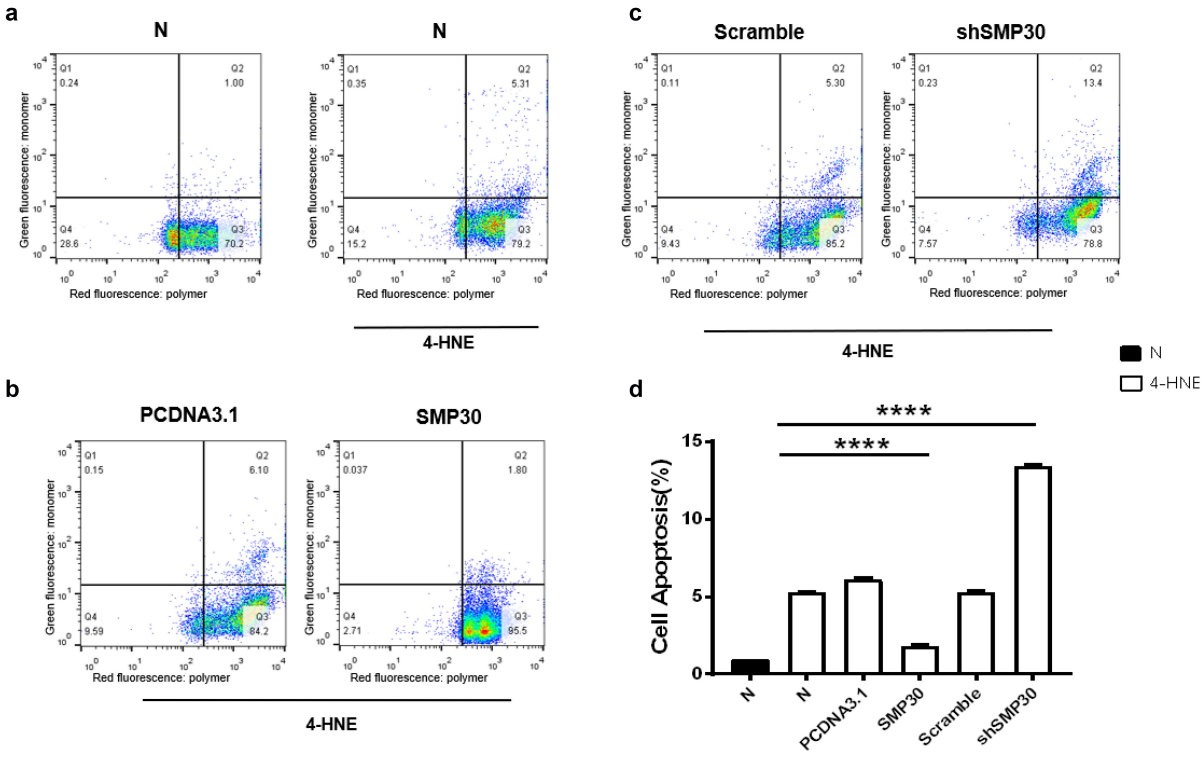

Figure 2. The effect of SMP30 on Mitochondrial membrane potential of HLECs treated with 4-HNE.

3.3. Overexpression of SMP30 restores the mitochondrial metabolism under 4-HNE condition

In order to explore the relationship between SMP30 overexpression and the mitochondrial function of HLECs treated with 4-HNE, we measured the OCR value of mitochondrial respiratory function in SMP30 overexpression group with 4-HNE inducement. As shown in Figure 3, after the cells were transfected with SMP30, OCR (Figure 3(a)) was used in determining the mitochondrial respiration functions of each group under 4-HNE condition. Mitochondrial-related indicators in HLECs exposed to 4-HNE conditions, including basal OCR (Figure 3(b)), ATP production (Figure 3(c)), maximum (Figure 3(d)), and spare respiratory capacity (Figure 3(e)) decreased. In summary, these metabolic characteristics indicate that SMP30 positively regulates mitochondrial oxidation in HLECs.

(a) Representative traces identifying the pattern of OCR in mitochondria stress tests after the injections of reagents [oligomycin, carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone (FCCP), and rotenone and antimycin A (RAA)] during the Seahorse analysis. (b) Basal OCR. (c) Maximal respiration. (d) Spare respiratory capacity in cells. (e) ATP production. *P < 0.05

3.4. Knockdown of SMP30 dysregulates mitochondrial bioenergetic profiles under the 4-HNE condition

In order to clarify the relationship between the knockdown of SMP30 and the mitochondrial function of HLECs treated with 4-HNE, we measured the OCR value of mitochondrial respiratory function in SMP30 knockdown group in 4-HNE treated HLECs group. As shown in Figure 4, after the cells were transfected with shSMP30, OCR (Figure 4(a)) was used in determining the mitochondrial respiration functions of each group under 4-HNE condition. Mitochondrial-related indicators in HLECs exposed to 4-HNE conditions increased, including basal OCR (Figure 4(b)), ATP production (Figure 4(c)), maximum (Figure 4(d)), and spare respiratory capacity (Figure 4(e)). In summary, these metabolic characteristics indicate that shSMP30 negatively regulates mitochondrial oxidation.

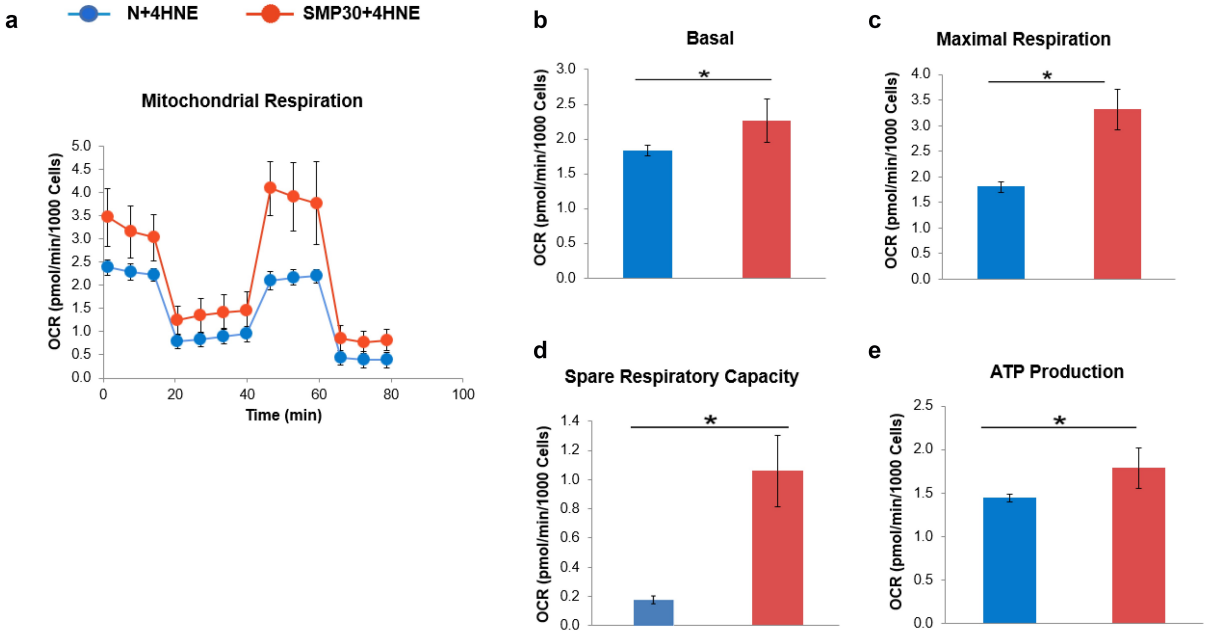

Figure 3. SMP30 affects the mitochondrial bioenergetic profiles under the 4-HNE condition.

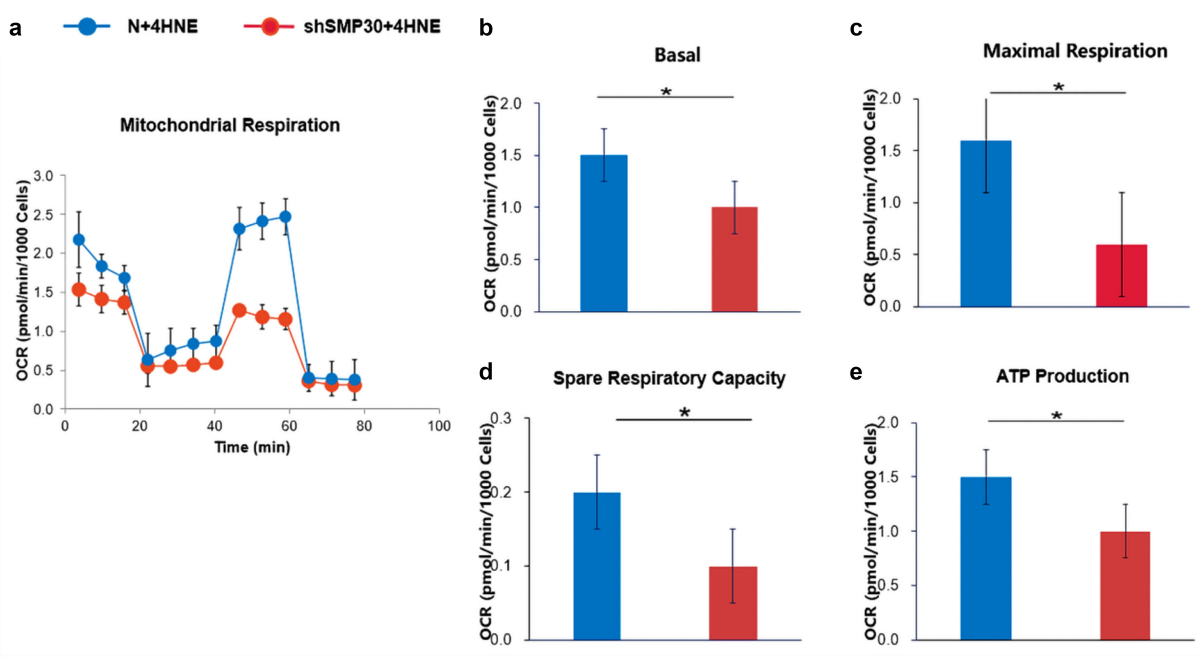

Figure 4. Knockdown of SMP30 can inhibit mitochondrial bioenergetic profiles under the 4-HNE condition.

(A) Representative traces identifying the pattern of OCR in mitochondria stress tests after the injections of reagents [oligomycin, carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone (FCCP), and rotenone and antimycin A (RAA)] during the Seahorse analysis. (B) Basal OCR. (C) Maximal respiration. (D) Spare respiratory capacity in cells . (E) ATP production. *P < 0.05

3.5. Effects of SMP30 on the cell morphology of HLECs treated with 4-HNE

To explore the effect of SMP30 on the cell morphology in HLECs treated with 4-HNE, we stained the cells in each group with H&E. H&E staining results showed that N group (Figure 5(a)) had full cell morphology, uniform cytoplasmic staining, round nuclei, and abundant cytoplasm; N + 4-HNE group (Figure 5(a)), PCDNA3.1 + 4-HNE group (Figure 5(b)), and scramble + 4-HNE group (Figure 5(c)) had reduced cell volumes, dense cytoplasm, and enhanced eosinophilic staining; shSMP30 + 4-HNE (Figure 5(c)) presented that the number of nucleus contraction increased significantly, which indicated that the knockdown of SMP30 inhibited the morphology of HLECs. Untreated cells were large and full, cytoplasmic staining was uniform, and the nucleus was round as well. After 4-HNE stimulation, the characteristic apoptotic changes, such as smaller, chromatin margination, nuclear hyperchromatism, pyknosis, etc were observed in the cells. The SMP30 + 4-HNE group (Figure 5(b)) had less apoptosis, and the overexpression of SMP30 significantly enhanced the morphology of the HLECs. As shown in Figure 5(d), the viability of normal cells was 0.98 ± 0.21. The N, PCDNA3.1, and SMP30 groups treated with 4-HNE had morphology scores of 0.36 ± 0.07, 0.39 ± 0.08, and 0.75 ± 0.12, respectively. The difference between the SMP30 and N + 4-HNE groups was statistically significant (F = 13.19, P = 0.016). SMP30 significantly upregulated the morphology of HLECs treated with 4-HNE. The cell morphology scores of the scramble and shSMP30 groups were 0.42 ± 0.08 and 0.17 ± 0.05, respectively. The difference between the shSMP30 and N groups was statistically significant (F = 13.19, P = 0.039), indicating that knockdown of SMP30 can significantly reduce the survival of HLECs.

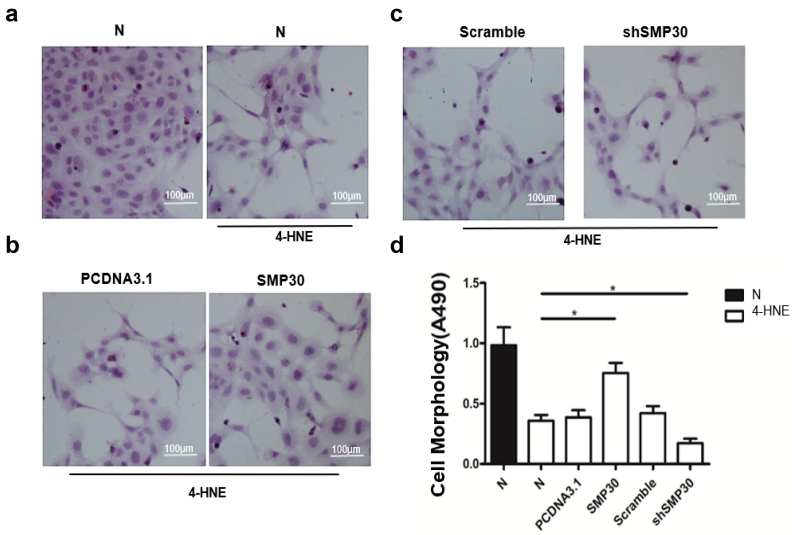

Figure 5. The effects of SMP30 on the morphology of HLECs treated with 4-HNE and the observation results of hematoxylin-eosin staining (H&E).

(A) The N group showed regular round and normal nuclei, and the N+4HNE group showed irregular round and fewer nuclei. (B) The SMP30 + 4-HNE group had less apoptosis than pcDNA3.1 group. (C) The shSMP30 + 4-HNE group showed typical characteristics of apoptosis compared to the shSMP30 group. (D) Bar chart showed cell morphology, with N+4HNE group as a control. *P < 0.05.

3.6. Effects of SMP30 on ROS production in HLECs treated with 4-HNE

To explore the effect of SMP30 on ROS production in 4-HNE treated HLECs, we performed DCFH-DA staining and flow cytometry in each group of cells. DCFH-DA test results showed that the brightness of ROS staining in the SMP30 group was significantly lower than that in the N group treated with 4-HNE (Figure 6(a-b)), indicating that the overexpression of SMP30 can reduce the production of ROS in HLECs. However, the ROS stain of the shSMP30 group was significantly stronger than that of the N group (Figure 6(a-c)), indicating that the decrease in SMP30 expression can stimulate increase in ROS production in HLECs. As shown in Figure 6(d), the production of ROS in the N group was 1758.41 ± 833.07. The production of ROS in the N, pcDNA3.1, and SMP30 groups treated with 4-HNE were 7814.59 ± 1547.81, 8659.28 ± 1110.13, and 4062.75 ± 627.23, respectively. The difference between the SMP30 and N groups was statistically significant (F = 24.17, P = 0.034). In HLECs, 4-HNE can induce ROS production, whereas SMP30 can obviously inhibit it. In the scramble and shSMP30 groups, the ROS production values were 8827.56 ± 1396.84 and 13,453.54 ± 270.59, respectively. The difference between the shSMP30 and N groups was statistically significant (F = 24.17, P = 0.016), indicating that the decreased expression of SMP30 can stimulate the production of ROS in HLECs.

To explore the effect of SMP30 on the total ROS of HLECs treated with 4-HNE, we subjected each cell group to flow cytometry. As shown in Figure 7, the total ROS activity in each group was detected. The ROS production values were 0 (N), 1200 ± 57.74 (N + 4-HNE), 1067 ± 66.67 (4-HNE+pcDNA3.1), 253.3 ± 3.333 (4-HNE+SMP30), 1133 ± 88.19 (4-HNE+Scramble), and 2533 ± 33.33 (4-HNE + shSNP30). The difference between the groups was statistically significant (F = 285.3, P < 0.0001). The result showed that the production of total ROS in the SMP30 group was lower than that in the N group treated with 4-HNE (F = 285.3, P < 0.0001). However, the production of total ROS in the shSMP30 group was higher than that in the SMP30

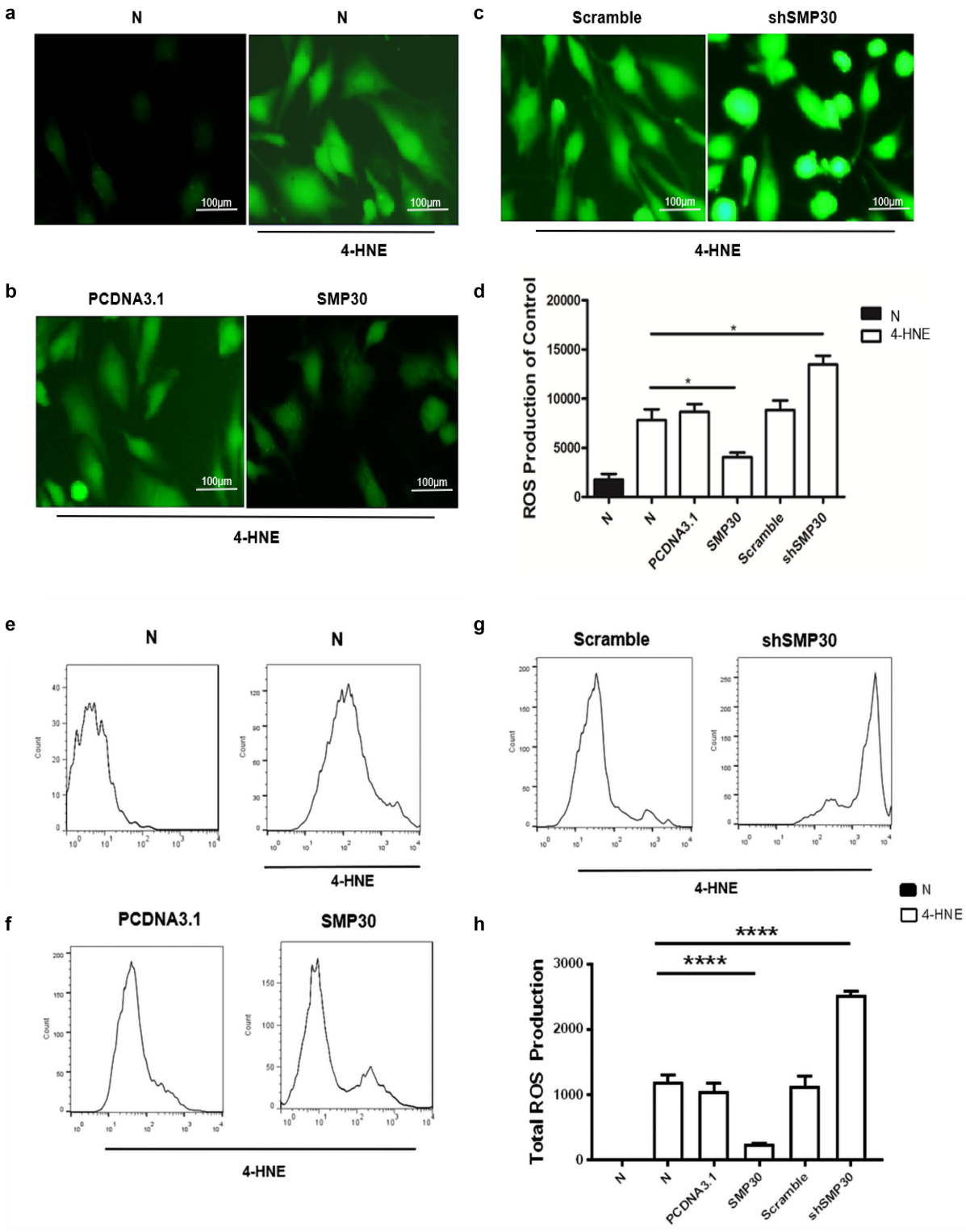

Figure 6. The effects of SMP30 on ROS production in HLECs cells treated with 4-HNE.
(a, b, and c) DCFH-DA test results showed that the ROS staining of the shSMP30 group was significantly stronger than that of the N groups treated with 4-HNE, while the brightness of ROS staining in the SMP30 group was significantly lower than that in the N group. (d) Bar chart showed ROS production, with N + 4HNE group as a control. (e) Flow cytometry was used to analyze the changes of intracellular ROS level of Normal group and N + 4-HNE group cells, (f) 4-HNE+pcDNA3.1 group and 4-HNE+SMP30 group, and (g) 4-HNE+Scramble group and 4-HNE+shSMP30 group by CM-H2CFDA. (h) Qualitative analysis of the yield of ROS in each group. *$P < 0.05$.

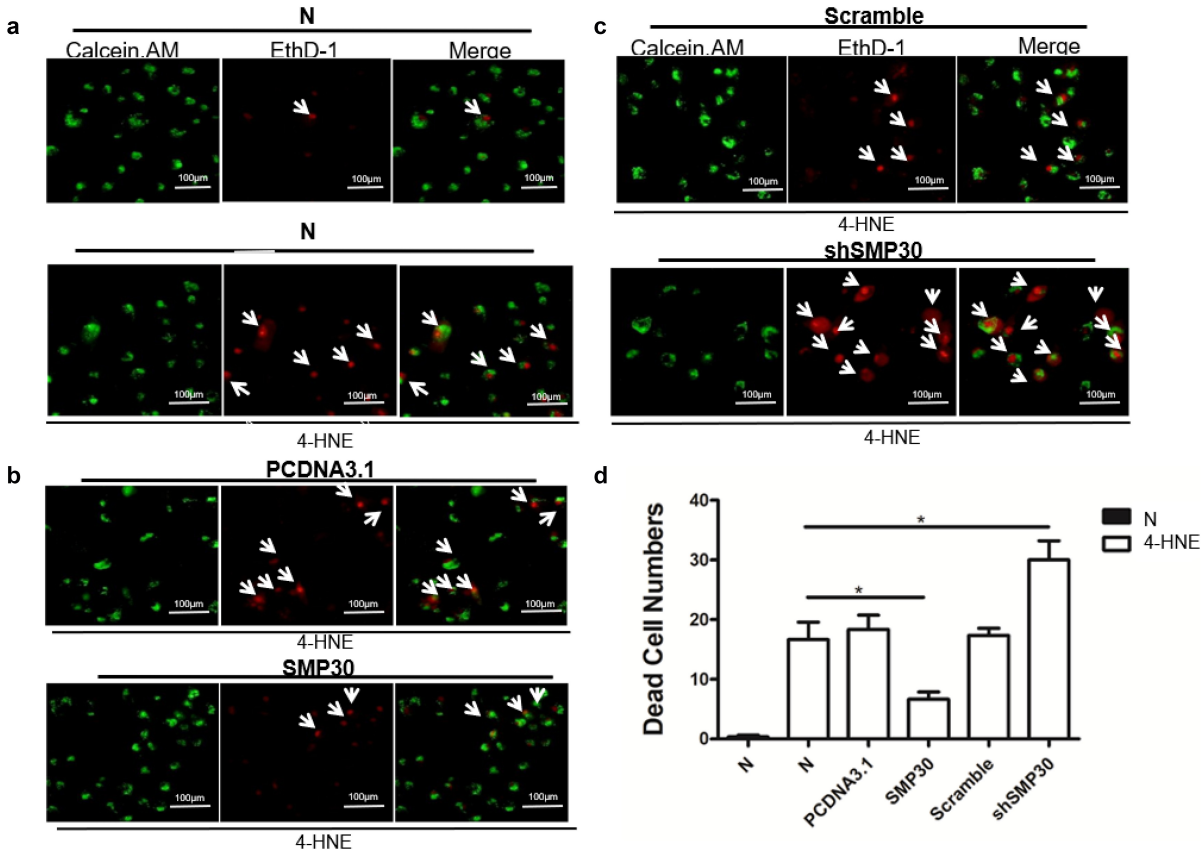

Figure 7. The oxidative stress damage of each group was marked by LIVE/DEAD staining.
(a, b, and c) LIVE/DEAD staining method was used to mark the cell survival status of each group. Live (green) and dead (red) cells. (d) Bar chart shows dead cell numbers of each group, with N + 4HNE group as a control. *P < 0.05.

group (F = 285.3, P < 0.0001), indicating that 4-HNE can induce total ROS production in HLECs, whereas SMP30 can obviously inhibit ROS production in HLECs.

3.7. Effects of SMP30 on oxidative stress damage of HLECs treated with 4-HNE

To explore the effect of SMP30 on oxidative stress damage in HLECs treated with 4-HNE, we performed live/dead staining in each group of cells. Under a fluorescent microscope, normally cultured cells had a stable morphology, uniform size, and uniform number and were exhibited as green living cells (Figure 7(a)). After 4-HNE treatment, the cells died, were red in color, and had a spot-like uneven distribution (Figure 7(a)). After treatment with 4-HNE, the number of red dead cells in the shSMP30 group (Figure 7(c)) increased significantly compared with that in the N group, whereas the number of red dead cells in the SMP30 group (Figure 7(b)) decreased significantly. The column chart showed the number of dead cells (Figure 7(d)): N group: 1.26 ± 0.04; N + 4-HNE group, 16.67 ± 4.11; pcDNA3.1 group, 18.33 ± 3.40; SMP30 group, 6.67 ± 1.70; scramble group, 17.33 ± 1.70; and shSMP30 group, 30 ± 4.55. The difference between the SMP30 and N groups was statistically significant (F = 23.03, P = 0.034), and the difference between the shSMP30 and N groups was statistically significant (F = 23.03, P = 0.037).

3.8. SMP30 upregulates Nrf2-dependent antioxidant protein expression

In order to study the antioxidant mechanism of SMP30, we studied the effect of SMP30 overexpression on the protein expression of Nrf2/Keap1 dependent antioxidant protection system under 4-HNE treatment. Nrf2 is a nuclear transcriptional factor, which controls more than 200 stress-associated genes, and Keap1 is a negative regulator of Nrf2 protein. The protein levels of Nrf2 and antioxidants,

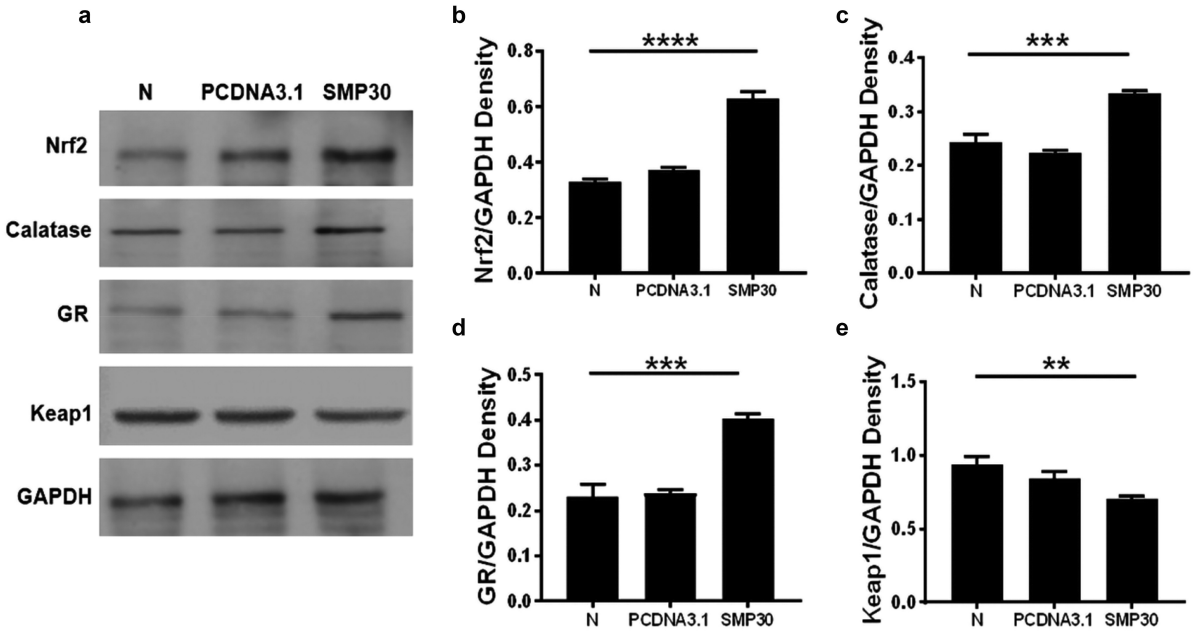

Figure 8. SMP30 upregulates Nrf2-dependent antioxidant protein expression.

including GR, and catalase are significantly increased in the SMP30 group, accompanied by a concomitant decrease in the level of Keap1. As shown in Figure 8 (b–d), the expression levels of Nrf2, catalase, and GR in SMP30 overexpression group were higher than those in the N and empty vector control groups ($F = 265, 110.4, 99.84$; $P < 0.0001, 0.0001, 0.0001$), whereas the expression of Keap1 was lower than that in the N and empty vector control groups ($F = 25.07$; $P = 0.0120$; Figure 8(e)), and the differences were statistically significant. SMP30 upregulates Nrf2, catalase, and GR expression and significantly downregulates the Keap1 protein.

(A) Expression enhancement of Nrf2 dependent antioxidant protection in SMP30 group. GAPDH was probed as a control. (B–E) Density measurement of Nrf2, catalase, GR, and Keap1. GAPDH was probed as a loading control. *$P < 0.05$.

4. Discussion

Lens epithelial cells are the most active sites for lens growth, differentiation, metabolism, synthesis, and transport. The occurrence and development of cataract are closely related to the physiological functions of and pathological changes in lens epithelial cells. Normal apoptosis is a necessary condition for the growth and development of the body and normal life activities, but abnormal or excessive apoptosis often causes lens diseases [2,24]. It is reported that different apoptotic pathways determine whether apoptosis occurs by acting on the mitochondrial membrane. Mitochondria play a key role in the process of apoptosis and even cell death. The apoptosis regulation mode centered on mitochondria is gradually understood and recognized by the public [25]. As the control center of apoptosis, mitochondrial dysfunction is closely related to the regulation of apoptosis. Due to the increase in oxidative stress, reactive oxygen species production and mitochondrial DNA mutation, the permeability transition pore located in the inner membrane of mitochondria can be opened. The components in the cytoplasm enter the mitochondria, resulting in a decrease in the membrane potential. Mitochondria release cytochrome c and apoptosis inducing factor on the inner membrane. Once cytochrome c enters the cytoplasm, it can initiate a series of apoptosis, activate caspase, and trigger apoptosis [26]. Lens epithelial cell apoptosis precedes lens opacity, suggesting that lens epithelial cell apoptosis may be an early event in cataract formation [13]. Oxidative damage is an important inducing factor for crystalline epithelial cell apoptosis. Oxidative stress is an

important risk factor for ARC. It is pointed out that the level of 4-HNE in cataract patients is significantly higher than that in normal control group [27]. 4-HNE is a marker of lipid peroxidation. Under physiological conditions, the concentration of 4-HNE in the plasma is usually extremely low. However, oxidative stress can increase its concentration 100-fold [28]. Lipid peroxidation is caused by the free radical attack of membrane lipids and produces a large number of reaction products, which are closely related to the mechanism of cataract [29–31]. Determining whether 4-HNE affects the level of apoptosis and functional changes in lens epithelial cells is important.

SMP30 is a regulatory factor related to apoptosis. It gradually increases with maturity and function and gradually decreases with organ function aging [32]. It has multiple protective effects, such as effects against antioxidant stress and apoptosis and proliferation regulation [33,34]. The decline in SMP30 marks the decline in cell function and the start of cell aging.

Studies on SMP30 in ophthalmology are few. SMP30 may be involved in the aging process of HLECs and the development of cataract [35,36]. The expression of SMP30 in the HLECs of ARC is weaker than that of transparent lens, indicating that the occurrence of ARC is accompanied by a decrease in the SMP30 content of lens epithelial cells. Our previous research confirmed that SMP30 overexpression downregulates the levels of Bax and cleave-caspase-3 but upregulates the Bcl-2 and pro-caspase-3 expression levels [37]. SMP30 may play a protective role in lens epithelial cells.

SMP30 plays an important role in antioxidant stress [38,39], and 4-HNE-induced oxidative damage is an important inducer of apoptosis in lens epithelial cells [7,8]. Oxidative stress refers to the excessive production of ROS and other macromolecules in the body exposed to harmful factors and may result in imbalance between oxidation and antioxidant systems and lead to cell and tissue damage. Oxidative damage to lens occurs before lens opacification [40,41]. ROS is an important factor in the apoptosis of lens epithelial cells [42,43]. Increase in ROS in lens epithelial cells damages the integrity of mitochondrial structure and function and then triggers the mitochondrial apoptosis pathway and starts cell apoptosis. Moreover, ROS can also affect the tricarboxylic acid cycle, damage the cell DNA, and lead to cell death.

The lens is an avascular tissue and undergoes anaerobic metabolism in a closed environment. The oxygen tension around the normal lens is less than 30 mmHg. As age increases or external factors change, the imbalance of the lens's antioxidant system generates excessive free radicals, subjecting lens epithelial cells to excessive oxidative stress that causes damage and weakening their defense. NO content in the aqueous humor of patients with ARC is significantly higher than that in normal people [44]. A large amount of NO produces oxidative stress damage to lens epithelial cells [45,46], and lens epithelial cells are the primary sites of oxidative damage. Transient oxidative stress can reduce the lens defensive function and cause lens epithelial cell apoptosis [47]. Given that 4-HNE can induce the generation of oxygen free radicals, 4-HNE-induced cell damage is essentially an oxidative stress. Our research found that SMP30 knockdown significantly upregulates the expression of ROS in HLECs, reduces cell morphology, and induces apoptosis. However, the overexpression of SMP30 can significantly downregulate the expression of ROS, upregulate cell morphology, and downregulate apoptosis of HLECs.

SMP30 has the function of an antioxidant [34,38,48], and the increased expression of SMP30 can improve the ability of LECs to resist oxidative stress. Li [35] reported that SMP30 may increase the antioxidant stress capacity of SRA01/04 cells by increasing SOD activity and reducing GSSG/T-GSH level. Change in SMP30 expression in LECs affects the level of oxidative stress to a certain extent.

The Nrf2/Keap1 signaling pathway is the most important antioxidant stress pathway in vivo [49,50]. Upon activation, it can regulate the expression of downstream antioxidant proteins and reduce the occurrence and degree of damage by oxidative stress to the body [51]. We speculate whether the antioxidant effect of SMP30 is related to the Nrf2/Keap1 signaling pathway and whether SMP30 enhances the antioxidant capacity of lens epithelial cells by activating the Nrf2/Keap1 signaling pathway.

Nrf2 is regulated by Keap1 and regulates the expression of antioxidant proteins by interacting

with the antioxidant response element ARE. Keap1 is the binding protein of Nrf2 in the cytoplasm. In general, Keap1 is 'off' and promotes the ubiquitination of Nrf2 in the cytoplasm until it is completely degraded. It inhibits the transfer of Nrf2 from the cytoplasm to the nucleus. When the cells are subjected to oxidative pressure, Keap 1 is 'on,' releasing Nrf2 to the nucleus and increasing the expression of downstream antioxidant proteins. The abnormal structure and function of the Nrf2/Keap1 system are closely related to the occurrence and development of cataract [52–54]. A large number of studies have shown that the function of the Nrf2/Keap1 antioxidant system in the lens epithelial cells of cataract patients is impaired [55–57]. Antioxidants, such as catalase and GR, play an important role in resisting oxidative response and protecting cells. A positive correlation has been found between the expression of Nrf2 and the mRNA level of these antioxidant genes, indicating that Nrf2 is necessary for the long-term induction of these genes [58]. The demethylation of Keap1 DNA promoter in LECs increases Keapl protein expression, which leads to a decrease in Nrf2 content and the inhibition of antioxidant enzyme gene transcription, thus promoting the occurrence and development of cataract.

Mitochondria are the main targets of hypoxic-ischemic injury [59]. Mitochondria are the organelles of cells that produce energy, and mitochondrial dysfunction is a marker of many diseases. Retrograde signal initiated by dysfunctional mitochondria can cause overall change in gene expression, thus changing cell morphology and function. This function is usually attributed to the disruption of important mitochondrial functions, such as ATP production, metabolic integration, calcium homeostasis, and apoptosis [60]. Dysfunctional renal mitochondria are the pathological mediators of diabetic nephropathy. The kidney is an organ with abundant mitochondria and high metabolism. Normal mitochondrial function can meet the needs of the kidney for a large amount of ATP. Diabetes affects mitochondrial function and changes the metabolic substrates produced by ATP, such as the transmission of fatty acids and oxygen. To meet the needs of ATP, diabetic patients change their metabolic fuel sources. This approach increases oxygen consumption, leading to renal anoxia and renal burden [61]. Other studies have shown that mitochondrial function changes under hypoxia, including the opening of bilayer membrane pores and uncoupling of mitochondrial electron transport chain, which can downregulate ATP and release calcium ion, cytochrome C, and apoptosis-inducing factor [62,63]. Mitochondria are the main consumers of oxygen and potential sources of ROS. In response to hypoxia, they exchange or modify the different subunits of the respiratory chain and regulate their metabolism, particularly reducing the citric acid cycle, which can affect the fate of hypoxic cells [8]. The present study showed that SMP30 affects mitochondrial bioenergetic profiles under 4-HNE condition and SMP30 positively regulates mitochondrial oxidation in HLECs. Decrease in SMP30 expression can reverse this phenomenon.

Our results showed that in the SMP30 overexpression group, Nrf2 increased, Keap1 decreased, antioxidation-related proteins, such as GR and catalase, increased, and mitochondrial oxidation increased in HLECs, suggesting that SMP30 protects lens epithelial cells from oxidative damage through the Nrf2/Keap1 signaling pathway and by regulating mitochondrial oxidation function and inhibiting the apoptosis of lens epithelial cells.

In conclusion, our study confirmed that the expression level of SMP30 is related to the regulation of apoptosis. SMP30 inhibits the accumulation of ROS caused by oxidative stress, may activate and protect the Nrf2/Keap1 signaling pathway, and regulate mitochondrial oxidation function, thereby protecting lens epithelial cells from oxidative stress-induced cell apoptosis. SMP30 may provide protection against ARC. To the best of our knowledge, this is the first study to demonstrate that SMP30 inhibits the apoptosis of HLECs by regulating mitochondrial oxidation. Therefore, SMP30 is a potential biological target for ARC prevention and treatment. In the future, we will further elaborate on the specific antioxidant mechanism of SMP30 in an animal model.

5. Conclusions

The expression of SMP30 in lens epithelial cells isolated from ARC was significantly lower than that in the control group, suggesting that the expression of SMP30 may be potentially related to the occurrence and development of cataract.

Subsequent functional experiments further confirmed that high expression of SMP30 could significantly inhibit the expression of ROS, restore mitochondrial functional homeostasis, protect the morphology of HLECs, and inhibit apoptosis. In terms of molecular mechanism, we showed that SMP30 overexpression could up-regulate the expression of antioxidant related proteins including Nrf2, GR, and catalase, meanwhile down-regulate the level of Keap1. Our study demonstrates that SMP30 may inhibit the accumulation of reactive oxygen species, regulate Nrf2/Keap1 signaling pathway and promote mitochondrial function for the first time. Since these functions can protect lens epithelial cells from oxidative damage, SMP30 is a potential therapeutic target for ARC therapy.

6. Contribution to the field statement

Cataract is the most common blinding eye disease in the world. No effective treatment other than the surgical removal of turbid crystals is currently available, and the pathogenesis of cataract is an extremely complex subject. Moreover, the etiology and pathogenesis of age-related cataract (ARC) are not completely clear. Recent studies have shown that the occurrence of cataract is a multifactorial process, in which oxidative damage is considered an important factor in the occurrence of ARC. This study found that SMP30 inhibits the production of ROS, activates the Nrf2/Keap1 signaling pathway, and promotes mitochondrial function. Given that these functions protect lens epithelial cells from oxidative damage, SMP30 is a potential therapeutic target for ARC treatment.

Abbreviations

SMP30: senescence marker protein30
ARC: age-related cataract
HLECs: human lens epithelial cells
HNE: 4-hydroxynonenal
ROS: reactive oxygen species
GR: glutathione reductase
TLECs: lens epithelial cells isolated from transparent lens
CLECs: lens epithelial cells isolated from ARC's lens
N: normal cell group
N + 4-HNE: normal cell group treated with 4-HNE
PCDNA3.1: SMP30 overexpression empty vector control group
SMP30: SMP30 overexpression group
Scramble: SMP30 knockdown empty vector control group
shSMP30: SMP30 knockdown group

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Author contributions

Lijie Dong designed the research; Xiaorong Li provided advice and guidance; He Teng, Yaru Hong, and Jingjing Cao performed the research; Hui Li, Xiaomin Zhang, Guoge Han, and Jing Sun drafted the paper; Yaru Hong, Kai Wen, and Amy Whelchel corrected article; FangTian provided fund assistance. All authors read and approved the final manuscript.

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