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ORIGINAL ARTICLE

Prolonged Exposure to High Glucose Induces Premature Senescence Through Oxidative Stress and Autophagy in Retinal Pigment Epithelial Cells

Chien-Chih Chiu[1,2,3,4,5] · Kai-Chun Cheng[6,7,12] · Yi-Hsiung Lin[8,9,10] · Chen-Xi He[1] · Yung-Ding Bow[11] · Chia-Yang Li[2] · Chang-Yi Wu[1,3] · Hui-Min David Wang[2] · Shwu-Jiuan Sheu[6,12]

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Abstract

Chronic hyperglycemia involves persistent high-glucose exposure and correlates with retinal degeneration. It causes various diseases, including diabetic retinopathy (DR), a major cause of adult vision loss. Most in vitro studies have investigated the damaging short-term effects of high glucose exposure on retinal pigment epithelial (RPE) cells. DR is also a severe complication of diabetes. In this study, we established a model with prolonged high-glucose exposure (15 and 75 mM exogenous glucose for two months) to mimic RPE tissue pathophysiology in patients with hyperglycemia. Prolonged high-glucose exposure attenuated glucose uptake and clonogenicity in ARPE-19 cells. It also significantly increased reactive oxygen species levels and decreased antioxidant protein (superoxide dismutase 2) levels in RPE cells, possibly causing oxidative stress and DNA damage and impairing proliferation. Western blotting showed that autophagic stress, endoplasmic reticulum stress, and genotoxic stress were induced by prolonged high-glucose exposure in RPE cells. Despite a moderate apoptotic cell population detected using the Annexin V-staining assay, the increases in the senescence-associated proteins p53 and p21 and SA-β-gal-positive cells suggest that prolonged high-glucose exposure dominantly sensitized RPE cells to premature senescence. Comprehensive next-generation sequencing suggested that upregulation of oxidative stress and DNA damage-associated

✉ Shwu-Jiuan Sheu
sjiuansheu@gmail.com

Chien-Chih Chiu
cchiu@kmu.edu.tw

Kai-Chun Cheng
pington64@gmail.com

Yi-Hsiung Lin
caminolin@gmail.com

Chen-Xi He
r060207@gap.kmu.edu.tw

Yung-Ding Bow
u109850001@kmu.edu.tw

Chia-Yang Li
chiayangli@kmu.edu.tw

Chang-Yi Wu
cywu@mail.nsysu.edu.tw

Hui-Min David Wang
davidw@dragon.nchu.edu.tw

[1] Department of Biotechnology, Kaohsiung Medical University, Kaohsiung 807, Taiwan

[2] Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan

[3] Department of Biological Sciences, National Sun Yat-Sen University, Kaohsiung 804, Taiwan

[4] Center for Cancer Research, Kaohsiung Medical University, Kaohsiung 807, Taiwan

[5] Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan

[6] Department of Ophthalmology, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan

[7] Department of Ophthalmology, Kaohsiung Municipal Siaogang Hospital, Kaohsiung 807, Taiwan

[8] Division of Cardiology, Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan

[9] Center for Lipid Biosciences, Kaohsiung Medical University Hospital, Kaohsiung 807, Taiwan

[10] Lipid Science and Aging Research Center, Kaohsiung Medical University, Kaohsiung 807, Taiwan

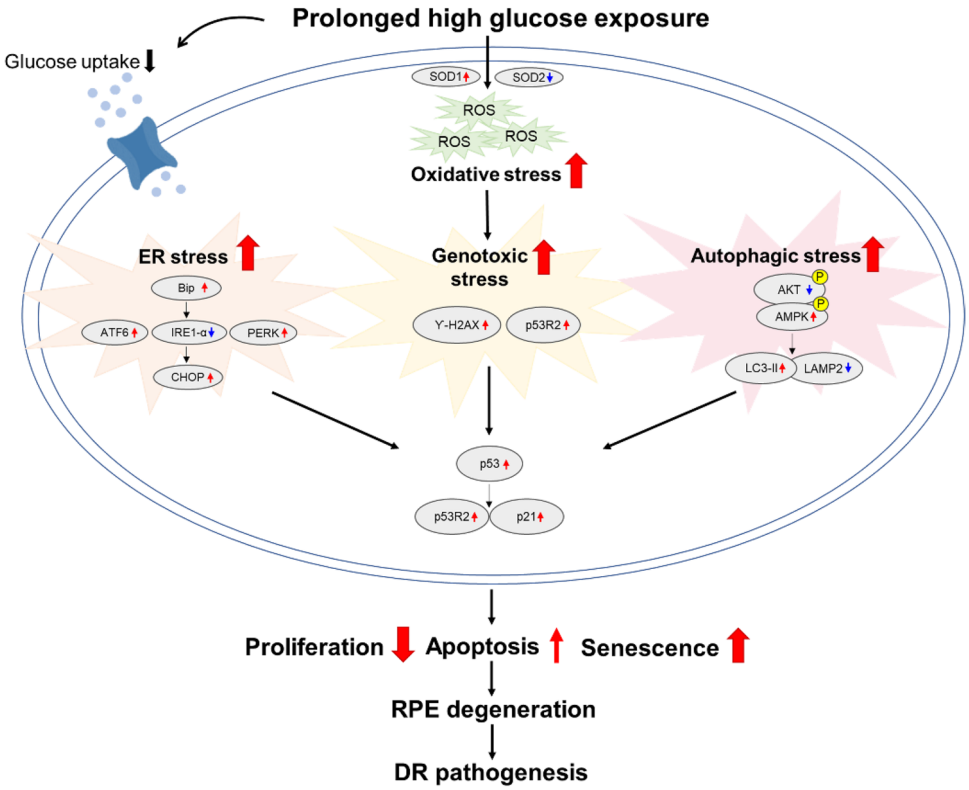
[11] Ph.D. Program in Life Sciences, College of Life Science, Kaohsiung Medical University, Kaohsiung 807, Taiwan

[12] Department of Ophthalmology, School of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan

pathways contributed to stress-induced premature senescence of ARPE-19 cells. Our findings elucidate the pathophysiology of hyperglycemia-associated retinal diseases and should benefit the future development of preventive drugs.

Graphical Abstract

Prolonged high-glucose exposure downregulates glucose uptake and oxidative stress by increasing reactive oxygen species (ROS) production through regulation of superoxide dismutase 2 (SOD2) expression. Autophagic stress, ER stress, and DNA damage stress (genotoxic stress) are also induced by prolonged high-glucose exposure in RPE cells. Consequently, multiple stresses induce the upregulation of the senescence-associated proteins p53 and p21. Although both apoptosis and premature senescence contribute to high glucose exposure-induced anti-proliferation of RPE cells, the present work shows that premature senescence rather than apoptosis is the dominant cause of RPE degeneration, eventually leading to the pathogenesis of DR.



Keywords Autophagy · Hyperglycemia · Oxidative stress · Premature senescence · Diabetic retinopathy

Introduction

Diabetes mellitus is the most common chronic disease with the highest incidence worldwide, and it is predicted that more than 69.3 million adults will suffer from diabetes mellitus in 2045 (Cole and Florez 2020). Both type 1 diabetes (insulin insufficiency) and type 2 diabetes (insulin resistance) cause chronic high blood glucose, which has been reported to correlate with diseases such as neuropathy, renal disease, heart disease, and diabetic retinopathy (DR) (Vargas et al. 2023). DR, a significant cause of visual impairment in developed countries and a worldwide public health issue, involves retinopathy with potential vision loss (American Diabetes 2011; Cheloni et al. 2019). Long-term high glucose worsens retinal pathogenesis, which is progressive and irreversible (Feldman-Billard et al. 2018; van Ballegooie et al. 1984). Several mechanisms have been reported to contribute to the development of DR, including oxidative stress, endoplasmic reticulum (ER) stress, autophagy, the AMP-activated protein kinase (AMPK) signaling pathway, and senescence (Adornetto et al. 2021; Chen et al. 2019; Dehdashtian et al. 2018; Farnoodian et al. 2016; Rosa et al. 2016; Shosha et al. 2018; Thounaojam et al. 2019; Xu and Ash 2016).

Previous studies have shown the effects of high glucose exposure on retinal cells, including human retinal vascular

endothelial cells, human retinal endothelial cells, and a retinal pigment epithelial (RPE) cell line (the ranges of used concentrations varied from 25 to 30 mM) (Chang et al. 2015; Foresti et al. 2015; Giurdanellaet al. 2020). Furthermore, Chen et al. (2012) used a comprehensive proteomic technique based on retinal cells to identify changes in protein expression and redox-associated retinal indicators using up to 100 mM d-glucose as a high glucose concentration. However, the above studies on high glucose concentrations focused on short-term-based high-glucose retinal cell models. In this study, we successfully established a prolonged high-glucose exposure cell model to mimic the pathophysiologic condition of RPE cells. With the assistance of the cell model, we investigated the pathways of prolonged high glucose exposure-induced degeneration of RPE cells in vitro, including oxidative stress, ER stress, autophagy modulation and stress-induced senescence phenotype. The possible mechanism underlying long-term high glucose exposure-induced pathogenesis in retinal degeneration was discussed.

Materials and Methods

Cell Culture

ARPE-19 cells were purchased from BCRC (#60383, Bioresource Collection and Research Center, Hsinchu, Taiwan). The cells were cultured in medium at a ratio of 3:2 Dulbecco's modified Eagle's medium (DMEM) with high glucose/F12 (Himedia, Mumbai, India) supplemented with 8% (v/v) fetal bovine serum, penicillin, and streptomycin in an incubator with 5% CO_2 at 37 °C. The cells were cultivated for two months with exogenous glucose treatments (15 and 75 mM).

Glucose Uptake Assay

2-NBDG (2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose) is a fluorescent analog of D-glucose, and the fluorescence intensity of intracellular 2-NBDG is proportional to glucose uptake (Zou et al. 2005). In 6-well culture plates, 1×10^4 cells were seeded. At 37 °C, the cells were treated for 1 h with a 2-NBDG glucose uptake assay kit (BioVision #K682). The fluorescence intensity of 2-NBDG was observed using an epifluorescence microscope and quantitated using an Accuri™ C6 flow cytometer with excitation/emission wavelengths of 475/550 nm.

Cell Proliferation Assay

A 96-well cell culture plate was used, and 2×10^4 cells were seeded per well. A Cell Counting Kit-8 (CCK-8) (IMT Formosa Co., Ltd., Taipei, Taiwan) assay was then used to assess cell proliferation. In a 96-well plate, 10 µl of CCK-8/well was added, and the plates were incubated for 2 h at 37 °C. To test cell viability, the absorbance at 450 nm was measured and calculated as a percentage of the control level. The cell viability was calculated using the mean optical density (OD) readings from six wells for each treatment.

Colony Formation Assay and Reactive Oxygen Species Scavenging Assessment

Five hundred ARPE-19 cells were seeded on a 12-well culture plate and grown for 24 h at 37 °C with 5% CO_2. Afterward, the cell culture medium was refreshed every two days until day 10. Afterward, the cells were fixed for 10 min with 4% paraformaldehyde (PFA) before being stained for 1 h with 1 × Giemsa stain (catalog number: 1.09204.1000, Merck, Darmstadt, Germany). The cells were imaged using a light plate after being washed in 1 × PBS. Finally, Image-Pro Plus v4.5 software (Media Cybernetics, Silver Spring, MD, USA) was used to quantitate the colonies. For the reactive oxygen species (ROS) scavenging assessment, medium with the indicated concentrations of *N*-acetylcysteine (NAC; Sigma–Aldrich, St. Louis, MO, USA) was freshly added to the prolonged high glucose (75 mM)-exposed cells every two days. To assess high glucose-exposed cells in response to oxidative stress, all groups of ARPE-19 cells were treated with 0.1 mM hydrogen peroxide (H_2O_2) for three hours. Afterward, the cells were refreshed with H_2O_2-free medium. Both of the above assessments followed the procedures described above, including the time of medium refreshment, cell fixation, and cell staining.

Western Blotting

Cells were lysed with 1 × RIPA solution (Billerica MA, USA), and the OD of the sample lysates was measured at 595 nm using a BCA protein assay kit (Pierce, Rockford, IL, USA). The samples were boiled before being resolved using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After that, the proteins were electrotransferred to a PVDF membrane. The membrane was blocked with 5% skimmed milk before being incubated with primary antibodies in 1 × TBS-T overnight at 4 °C. The membrane was then rinsed with TBS-T and incubated with secondary antibodies against the primary antibodies. The signals of specific proteins on the PVDF blots were detected using an enhanced chemiluminescence kit (Advansta Inc. Menlo Park, CA, USA).

Assessment of Intracellular ROS

Briefly, 8×10^4 cells were seeded into six-well plates at 37 °C in a 5% CO_2 incubator overnight. Then, the medium was refreshed, and the cells were incubated with 10 µM

2′,7′dichlorodihydrofluorescein diacetate (DCFDA) as a probe (Molecular Probes Inc., Eugene OR, USA) and dihydroethidium (DHE) (Invitrogen™, D11347) at 37 °C for 15 min. The fluorescence intensity of DCFDA and DHE was measured using an epifluorescence microscope and quantified using flow cytometry with an Accuri™ C6 with excitation/emission wavelengths of 485/525 nm and 518/606 nm, respectively.

Apoptosis Assessment

A total of 1×10^5 ARPE-19 cells were seeded in a 60-mm petri dish and cultured for 24 h. Then, the cells were harvested with trypsin. The procedure was performed according to the manufacturer's instructions (Strong Biotech Corporation, #AVK005, Taipei, Taiwan). In brief, the cells were stained with 10 µg/ml Annexin V-conjugated fluorescein isothiocyanate (#AVK005, Strong Biotech Corporation, Taipei, Taiwan) and 1 µg/ml 7AAD (#11397, Cayman) and incubated for 30 min at 37 °C. Then, the cells were analyzed using an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo v7.6.1 software (TreeStar, Inc., Ashland, OR, USA).

mRNA Extraction and Qrt-PCR Assessment

Total RNA was isolated with a Nucleospin® RNA kit (Macherey-Nagel, USA). Briefly, the samples were lysed with a pestle in lysis buffer. The lysate was added to the RNA column and centrifuged to isolate nucleotides. Subsequently, DNase was utilized to degrade the DNA and purify the RNA sample. Finally, the column was transferred to a new 1.5 ml tube, and the RNA sample was eluted with RNase-free H_2O. The isolated RNA samples were stored at − 80 °C. Complementary DNA (cDNA) was synthesized using a high-capacity RNA-to-cDNA kit (Applied Biosystems, USA). A 96-well quantitative polymerase chain reaction (PCR) thermal cycler was used for gene expression analysis.

Profiling of Gene Expression Using Next-Generation Sequencing and Bioinformatics Analysis

RNA library construction and sequencing were performed by Biotools Biotechnology Co. (Taipei, Taiwan). The detailed procedure was described previously (Lin et al. 2023). Briefly, a TruSeq™ RNA LT Sample Preparation kit (Illumina Inc., San Diego, CA, USA) was used with the total RNA for library construction according to the manufacturer's instructions. The magnetic beads were calibrated with oligo(dT) to collect mRNA from ARPE-19 cells in the indicated treatments. cDNA synthesis was performed, and the second strand was synthesized through nick translation. The cDNA library was prepared by the following processes: purification, terminal repair, A-tailing, ligation of sequence adaptors, size selection and PCR enrichment. The cDNA size in the library adapters was assessed, and PCR-based quantitation was performed. Sequencing of the RNA library was performed on an Illumina HiSeq 2500 instrument (Illumina, Inc., CA, USA), and the changes in gene expression were analyzed. Regarding biological pathway analysis, the differential gene results were screened, and downstream systematic bioassays were performed using the Ingenuity Pathway Analysis (IPA) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (https://www.genome.jp/kegg/kegg3a.html) to predict potential pathways.

SA-gal Staining

Glutaraldehyde was utilized to fix cells. The fixed cells were then stained with 1 mg/ml 5-bromo-4-chloro-3-indoyl-galactoside (X-gal, dissolved in a solvent composed of dimethylformamide, 40 mM citric acid, 2 mM magnesium chloride, 5 mM potassium ferrocyanide (II), 5 Mm potassium ferricyanide (III), 150 mM sodium chloride, 40 mM sodium phosphate, pH 6.0) for 24 h. The stained cells were washed with PBS, and the X-gal (green)-stained positive cells were assessed.

Detection of Lipofuscin Accumulation

Lipofuscin was stained with 0.1% Sudan Black B in 70% ethanol for 5 min after the cells were fixed with % PFA. To remove the excess dye, the cells were washed again with 70% ethanol. Nuclear fast red stain (0.1%, catalog number: 6409-77-4, Sigma, USA) was used to mark the nuclei of the cells. An inverted fluorescence microscope was used to observe the stained cells.

Animal Model

Eight-week-old B6. BKS(D)-lepr^{db}/J mice that exhibit an obese and diabetic phenotype were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in a temperature- and humidity-controlled environment with free access to food and water. The light:dark cycle was 12 h light:12 h dark. The experiments were approved and supervised by the Institutional Animal Care and Use Committee (IACUC) of Kaohsiung Medical University (IACUC approval number: 110169).

Immunofluorescence in Animal Model Tissue

The retinal tissues were embedded in paraffin after being treated with 4% paraformaldehyde overnight. Three-meter portions were cut from the tissue block. Following an overnight incubation with primary senescence-related antibodies

such RB antibody (SC-102, Santa Cruz) and P16 antibody (10883-1-1AP, Proteintech), the sections were exposed to secondary antibodies (Alexa Fluor 568 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG, both were purchased from Invitrogen, Carlsbad, CA, USA). The cell nuclei were stained with 1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI; 62248, Thermo Fisher Scientific, Waltham, MA, USA). With a magnification of 200×, an Olympus BX61 microscope (Olympus, Tokyo, Japan) was employed.

Statistical Analysis

The results are presented as the means ± SEMs. One-way analysis of variance (ANOVA) followed by the Mann–Whitney *U* test with two-tailed probability was used to assess group means. The statistical software SigmaStat (SPSS, Chicago, IL, USA) was used for data analysis. A *p*-value of less than 0.05 was considered to indicate statistical significance.

Results

Prolonged high-glucose exposure of ARPE-19 cells attenuated glucose uptake, proliferation, and clonogenicity. We preliminarily examined short-term exposure (24 h and 48 h) of ARPE-19 cells to exogenous glucose (+ 15, + 45, and + 75 mM) but found no significant difference compared with the control group of cells without exogenous glucose treatment (data not shown). Our results showed that prolonged glucose exposure reduced the capacity of cells to import glucose (Fig. 1A) and drastically reduced the ability of the cells to form colonies (Fig. 1B, C), and a flattened cellular morphology was observed (Fig. 1D). Additionally, the cellular viability was decreased (Fig. 1E). Furthermore, a decrease in AKT phosphorylation at Ser^{473} and Thr^{308} (Fig. 1F) suggested that prolonged high glucose exposure may impair AKT activation in ARPE-19 cells.

High glucose exposure led to an increase in the production of ROS in APRPE-19 cells. During high-glucose exposure,

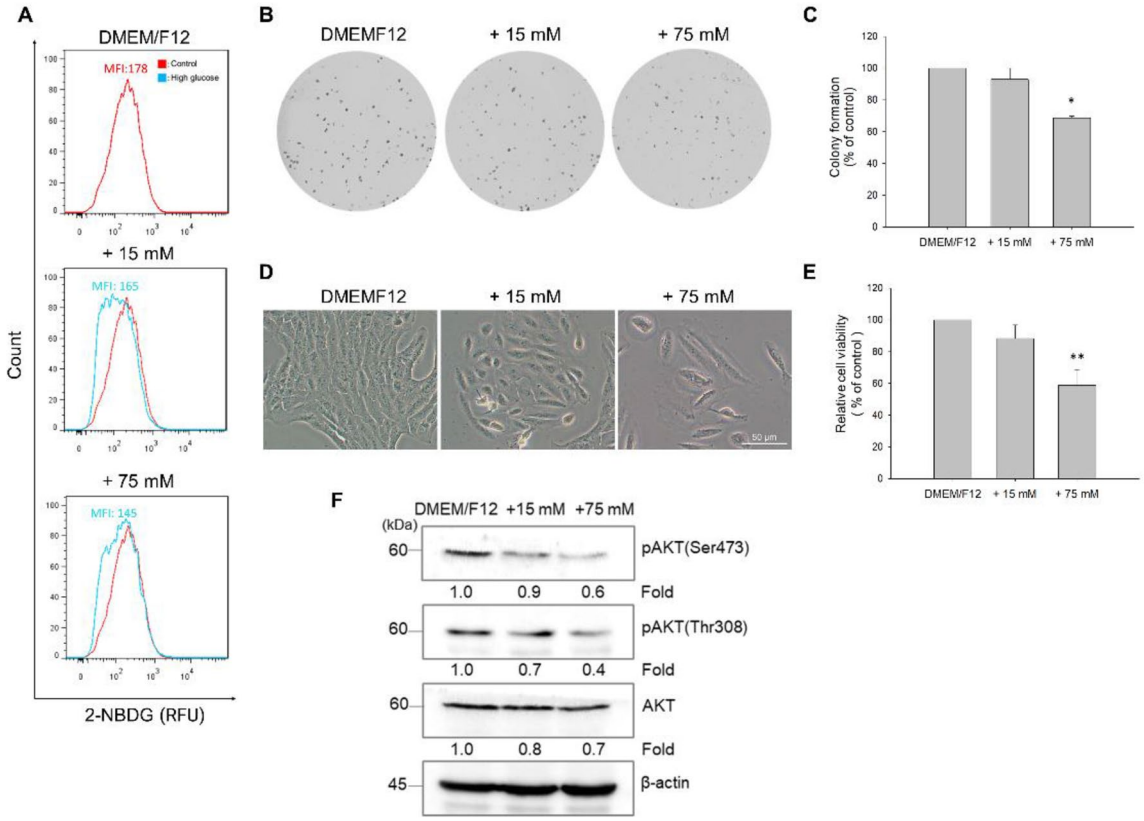
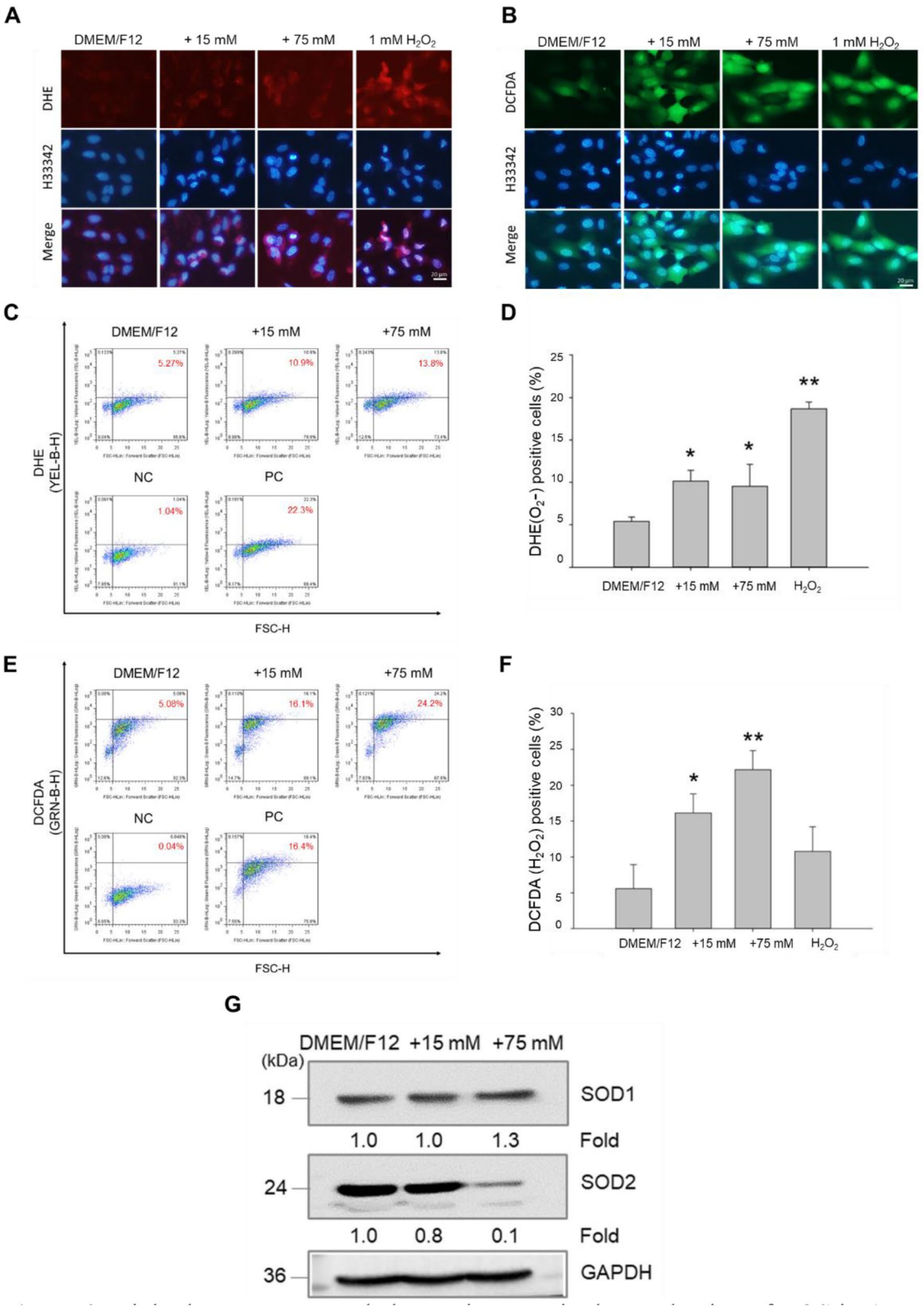


Fig. 1 Prolonged high-glucose exposure of ARPE-19 cells attenuated glucose uptake, proliferation, and clonogenicity. **A** Assessment of glucose uptake using flow cytometer-based staining of cells with 2-NBDG, a fluorescent deoxyglucose analog. **B** Representative results of colony formation of ARPE-19 cells following long-term high-glucose exposure. **C**. Quantitative analysis of **B**. Exposure to high glucose attenuated clonogenicity, an index of long-term proliferation in ARPE-19 cells. **D** Observed morphological changes. **E** Quantitative analysis of cell viability using the CCK-8 assay. **F** Changes in AKT phosphorylation in ARPE-19 cells after high-glucose exposure. The concentrations of exogenous glucose used for high-glucose treatment were 15 and 75 mM. *$p < 0.05$; **$p < 0.01$



ROS are produced due to oxidative stress and homeostatic imbalance (Robles-Rivera et al. 2020). To confirm this, we stained cells with the dyes DHE (to detect superoxide $O_2^{\cdot-}$) and DCFDA (to detect hydrogen peroxide H_2O_2) and found an increase in the level of ROS production (Fig. 2A–F). To further confirm this finding, we performed Western blotting,

◂**Fig. 2** High glucose exposure led to an increase in the production of ROS in APRPE-19 cells. **A**, **B** ROS production was significantly enhanced, as demonstrated by epifluorescence microscopy using the oxidation-sensitive fluorescent dyes DHE and DCFDA. The axis FSC-H vs ROS profiles (DHE and DCFDA) are presented to illustrate cell size and signaling. **C**, **D** Representative results of O_2^- assessment using flow cytometer-based DHE staining and quantitative analysis. **E**, **F** Representative results of H_2O_2 assessment using flow cytometer-based DCFDA staining and quantitative analysis. NC: Cells without dye staining. 2 mM H_2O_2 as a positive control. **G** Western blotting showed changes in ROS-related proteins, including SOD1 and SOD2. The concentrations of exogenous glucose used for high-glucose treatment were 15 and 75 mM. $^*p < 0.05$; $^{**}p < 0.01$

which showed decreased levels of SOD2, the hallmark of oxidative stress (Fig. 2G). These results show that there is a significant increase in the production of ROS in RPE cells following prolonged high-glucose exposure.

Our results showed that long-term exposure to a high glucose level increased ROS stress and attenuated the proliferation of RPE cells. Therefore, we further examined the effect of a ROS scavenger (NAC) on the proliferation of ARPE-19 cells, and the inhibitory effect of prolonged high glucose was significantly reversed by NAC treatment in a dose-dependent manner (Fig. 3A, B), suggesting that prolonged high glucose exposure attenuates the proliferation of RPE cells, which may be at least partially attributed to the increase in ROS production, an index of intracellular oxidative stress.

High Glucose Exposure Affects ER Stress and Autophagy in RPE Cells

High levels of blood glucose have been shown to cause the production of ROS in the mitochondria and increase a significant burden on the ER, which is responsible for the proper folding of nascent synthesized proteins (Pandey et al. 2019). When ER homeostasis is disrupted by factors such as oxidative stress, inflammation, and hyperglycemia, ER stress is induced (Pandey et al. 2019), leading to cell damage or cell death (Hsu et al. 2019). In addition to ER stress, the dysregulation or downregulation of autophagic flux, for example, by blocking the fusion of autophagosomes and lysosomes in cells, may contribute to the pathophysiological progression of DR (Aragones et al. 2020; Rosa et al. 2016).

Therefore, we further examined the changes in autophagy- and ER stress-associated proteins, and we found that high glucose stress increased the expression of the autophagic protein microtubule-associated protein 1A/1B light chain 3B (LC3B) but markedly decreased that of lysosome-associated membrane protein 2 (LAMP2), whereas no significant changes in p62 were observed (Fig. 4A). Apart from autophagy, we observed the accumulation of CHOP and ER stress-associated proteins (Bip/GRP78, the ER chaperone, PERK and ATF6) (Fig. 4B).

Effect of Prolonged High-Glucose Exposure on the Fate of RPE Cells

Next, we assessed the effect of high glucose exposure on ARPE-19 cells. For this, the cells were seeded in six-well plates overnight. The results showed that a moderate apoptotic population was detected (increase of approximately 6% compared with control cells (DMEM/F12) without high glucose exposure) using annexin V/7-AAD staining (Fig. 5A). In the colony formation assay, the high glucose-exposed cells were insensitive to H_2O_2 (Fig. 5B), which is a hallmark of senescent cells. Next, we performed senescence-associated galactosidase (SA-gal; X-gal, pH 6.0) staining, which showed that the cells adopted a senescence-like phenotype instead of undergoing apoptosis (Fig. 5C, D). To confirm the senescence-like stage, we performed Western blotting to detect the level of p21 protein, which is a senescence marker. We noted a significant increase in p21 protein expression. These results showed an increase in senescence but not apoptosis (Fig. 5E). Lipofuscin aggregates mainly constitute oxidized proteins and lipids, which are markers of cellular senescence (Georgakopoulou et al. 2013). Therefore, we next used lipofuscin staining to determine whether prolonged high-glucose exposure promotes senescence in ARPE-19 cells (Fig. 5F).

Next-Generation Sequencing Investigation of the Differentially Expressed Genes Involved in High-Glucose Stress Signaling in RPE Cells

In this study, we were able to demonstrate the cellular stress signal transduction pathways in retinal cells that are related to abnormal glucose levels. Figure 6A shows a heatmap of gene expression differences between the normal RPE cells and the cells in the high-glucose group. Red indicates increased gene expression, while blue indicates decreased gene expression. Figure 6B shows the differentially expressed genes (DEGs) between the normal group and the high-glucose group, with a total of 1635 genes involved in high-glucose stress. We further analyzed the DEGs using IPA and found that 94 upstream regulators were affected and regulated in RPE cells. Among them, 56 regulators were inhibited due to high-glucose stimulation, while 38 regulators exhibited activated expression due to high-glucose stimulation (Fig. 6C). We further analyzed the DEGs involved in canonical pathways, and the impacted pathways were ranked and displayed from high to low. We found that 72 regulated genes were related to senescence signaling (Fig. 6D). After analyzing the related diseases and biofunctions, we found that many genes related to cell death were activated; in contrast, genes related to cell survival were inhibited or suppressed (Fig. 6E, F). This evidence supports the gene regulation results indicating that high glucose is

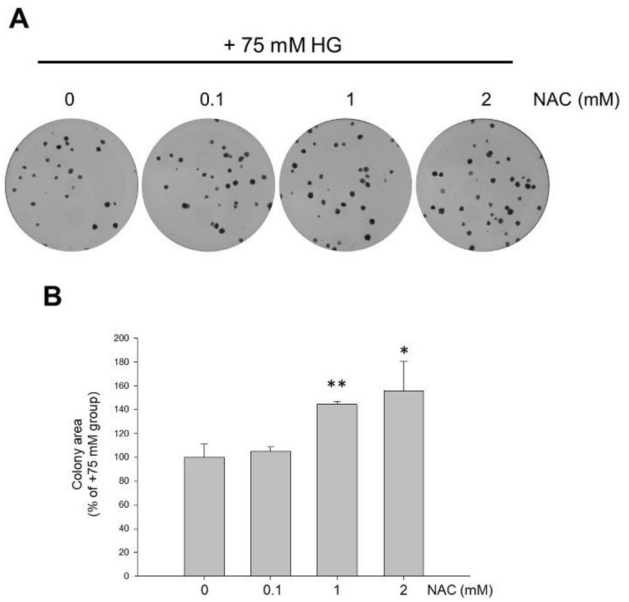


Fig. 3 ROS scavenging reversed the long-term proliferation arrest of RPE cells induced by high glucose exposure. A colony formation assay was performed to assess the impact of long-term exogenous high-glucose exposure (75 mM) on the proliferation of ARPE-19 cells. **A** High glucose exposure inhibited the proliferation and clonogenicity of ARPE-19 cells, whereas NAC reversed the effects caused by high glucose exposure and induced proliferation arrest in a dose-dependent manner. **B** Quantitative analysis of the colony formation assay results. The indicated concentrations of NAC, a ROS scavenger, were used. The concentration of exogenous glucose used for high-glucose treatment was 75 mM. $*p < 0.05$; $**p < 0.01$

detrimental to the survival of RPE cells. Notably, regulators related to aging were greatly affected.

KEGG Investigation of High-Glucose Stress-Initiated RPE Cell Senescence and Cell Cycle Distribution Modification

Our analysis revealed the most enriched canonical pathways of the regulated genes when retinal cells were subjected to high-glucose stress. Among them, we found that genes related to the senescence pathway were greatly activated and highly expressed. Therefore, KEGG software was further applied to analyze the signal transduction pathways that are currently known to be related to the senescence pathway. As shown in Fig. 7A, the KEGG pathway enrichment study by IPA analysis suggests high glucose-related underlying cellular senescence-activated signaling. Regulators with pink indicated the increasing/upregulation of gene expression, and orange indicated the predicted increased/upregulated gene expression response to high glucose incubation. Otherwise, the green regulators (decreased/downregulated) and blue (predicated decreased/downregulated) gene expressions were marked by high glucose incubation. Among them, the upstream regulator CDK inhibitor p21 was significantly elevated at both the gene and protein levels. In addition, the key factors of senescence, TP53 and RB, were both found to be upregulated by high glucose and initiated cellular senescence sequentially (Fig. 7A). Furthermore, KEGG pathway analysis of cell cycle regulation was also shown to suggest the cell cycle response of ARPE-19. The

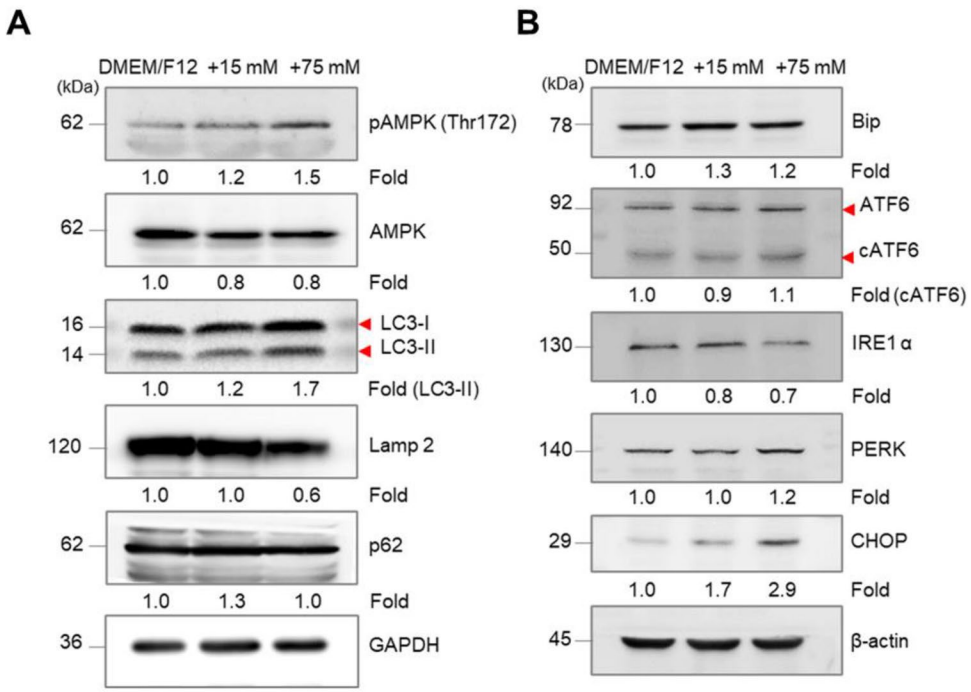


Fig. 4 High glucose exposure affects the expression of proteins related to autophagy and ER stress in RPE cells. **A** Western blots showing a decrease in AMPK and cleavage of LC3B after high-glucose treatment. LC3B was modified to LC3B-I and LC3B-II, suggesting that autophagy was initiated after high-glucose exposure and decreased LAMP2 expression, whereas no significant changes in p62 protein were observed. **B** Western blots showing the accumulation of CHOP, Bip, PERK, ATF6, and ER stress-associated proteins in ARPE-19 cells. The concentrations of exogenous glucose used for high-glucose treatment were 15 and 75 mM. GAPDH and β-actin were used as internal controls

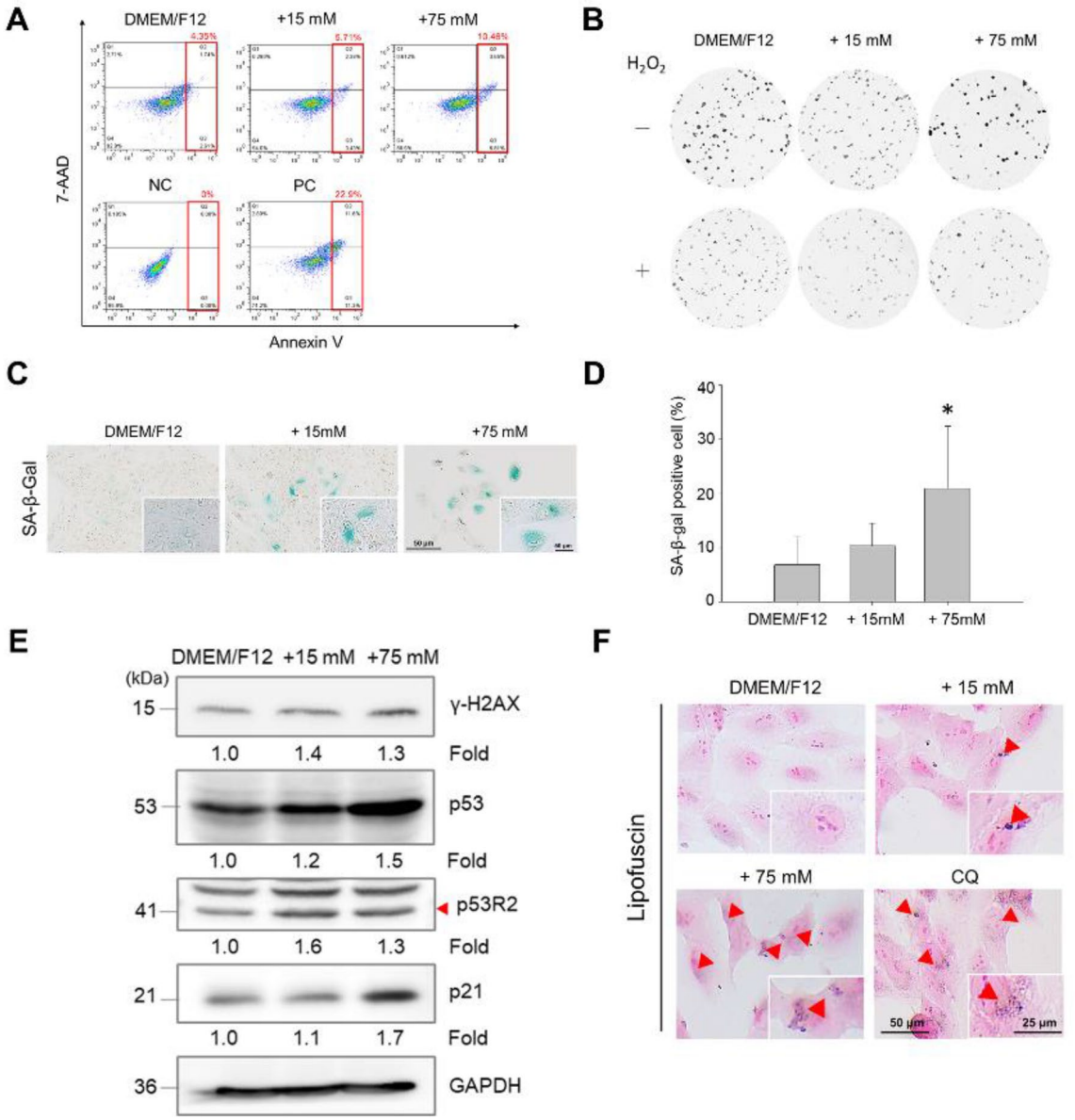


Fig. 5 Effect of prolonged high glucose exposure on the fate of RPE cells. ARPE-19 cells were seeded on a 6-well plate overnight. Afterward, **A** apoptosis was observed using annexin V/7-AAD staining. Negative control (NC), cells without staining. Positive control (PC): 5 mM H_2O_2 overnight. **B** Apoptotic cell death was not significantly affected by high glucose exposure in ARPE-19 cells. Surprisingly, the colony formation assay showed that high glucose-exposed ARPE-19 cells were resistant to H_2O_2. **C**, **D** SA-gal (X-gal, pH 6.0)-positive cells showed a senescence-like phenotype due to high-glucose exposure, suggesting that long-term glucose exposure causes stress and hence premature senescence. **E** Western blot analysis showed increases in the levels of p53 and p21, which are senescence-associated proteins. In addition, upregulation of p53-controlled ribonucleotide reductase (p53R2), a factor associated with p53-dependent senescence, was also observed. **F** Senescence assessment using Sudan-Black-B-specific staining of lipofuscin. The red arrows indicate lipofuscin aggregation. The concentrations of exogenous glucose used for high-glucose treatment were 15 and 75 mM. $*p < 0.05$

results suggest that the downregulation of cyclin D/F and CDK2/4/6 could induce cell cycle arrest in the G1 and S phases, and the results could also reflect the fact that high glucose incubation impaired ARPE-19 cell proliferation (Fig. 7B). These regulatory crosstalk networks revealed for the first time the biological impact of RPE cells under high-glucose stress.

High Glucose Exposure Induces the Senescence of Retinal Tissues

Although it has been hypothesized that both cellular senescence and apoptosis contribute to the degeneration of retinal tissue and, eventually, to retinopathy. Using a diabetic mouse model, Shosha et al. found that diabetes causes early

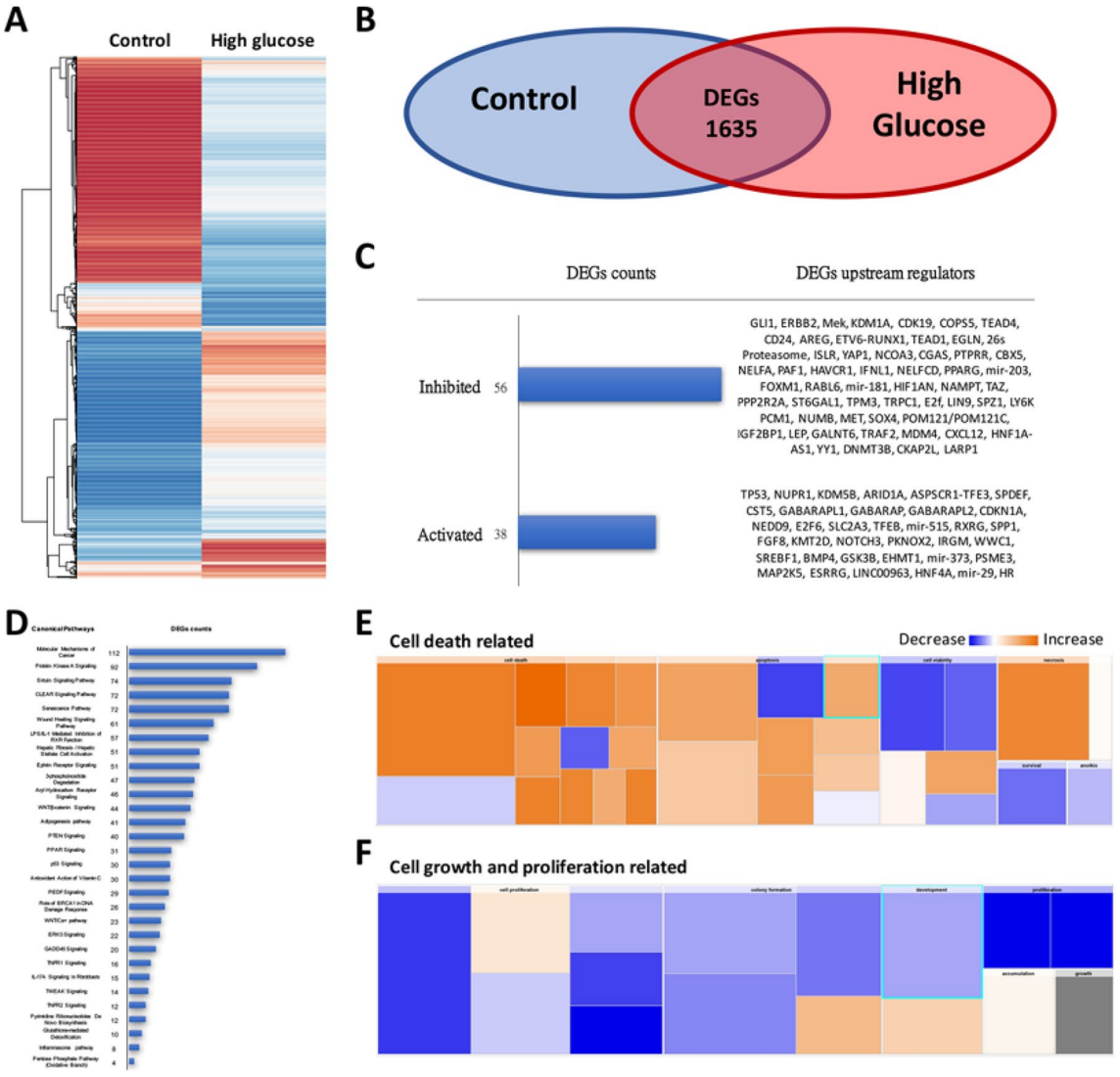


Fig. 6 Expression profiling of genes using RNA sequencing analysis. **A** Heatmap of the genes associated with control and high glucose-treated RPE cells. **B** Control and high glucose-treated RPE DEGs. **C** Upstream regulators of DEGs that were inhibited or activated by high-glucose stimulation. **D** Canonical pathways in which the high glucose-treated RPE cell DEGs were involved. DEGs associated with the diseases and biofunctions of **E** cell death and **F** cell growth and proliferation were analyzed

senescence of endothelial cells in retinal tissue (Rojas et al. 2017; Shosha et al. 2018). Based on the findings of our study, however, the fate of RPE cells exposed to prolonged high glucose appears to be premature senescence rather than apoptosis, which may be the culprit that contributes to the degeneration of diabetic retinopathy. In cell-based (Rojas et al. 2017) and mouse-based models of DR pathogenesis, premature senescence has also been demonstrated. Senescent cells generate high quantities of ROS and oxidative DNA damage. DNA damage, telomere erosion, and oxidative stress induce cell cycle arrest via the $p16^{INK4a}$/Rb and $p53/p21^{Cip1}$ pathways, which in turn induce cellular senescence (Chen et al. 1995; Song et al. 2005). Immunohistochemical staining consistently detected an increase in the senescence markers p16 and Rb in the retinal tissue of diabetic mice, indicating that continuous glucose exposure causes retinal tissue senescence (Fig. 8).

Discussion

RPE degradation is gradual and irreversible in people with long-term DR. Recent research suggests that hyperglycemia-induced oxidative stress may be the cause of ocular degeneration and autophagy dysfunction during the progression of DR (Cecilia et al. 2019; Duraisamy et al. 2018; Farnoodian et al. 2016; Li et al. 2019). Despite the importance of chronic hyperglycemia as a health issue, most studies

on high glucose exposure have focused on short-term retinal cell models (Chang et al. 2015; Foresti et al. 2015) to mimic the pathophysiological impact of hyperglycemia on the degeneration of RPE cells, which may not reflect the pathophysiological outcome.

In addition, DMEM/F12 is widely used as a growth medium to maintain ARPE-19 cells (ATCC# CRL-2302™) for in vitro studies on diabetes, and a physiological glucose concentration of 5.5 mM is often used for studies on diabetic complications. However, it has been reported that ARPE-19 cells in low-glucose medium have a reduced growth rate and altered morphology (Heimsath et al. 2006). Short-term exposure to DMEM/F12 as the growth medium (approximately 17 mM glucose) does not change the cell morphology or survival rate of ARPE-19 cells but increases intracellular ROS levels (Haranahalli Shivarudrappa et al. 2019), which are unsuitable for long-term maintenance. Therefore, we used standard medium (17 mM glucose) as a control for a long time period to avoid the morphological alterations caused by prolonged exposure of ARPE-19 cells to low-glucose medium. In this study, we established a model via prolonged culture with exogenous glucose (15 and 75 mM) (Haranahalli Shivarudrappa et al. 2019) to mimic the pathophysiologic conditions of RPE tissues of patients with hyperglycemia.

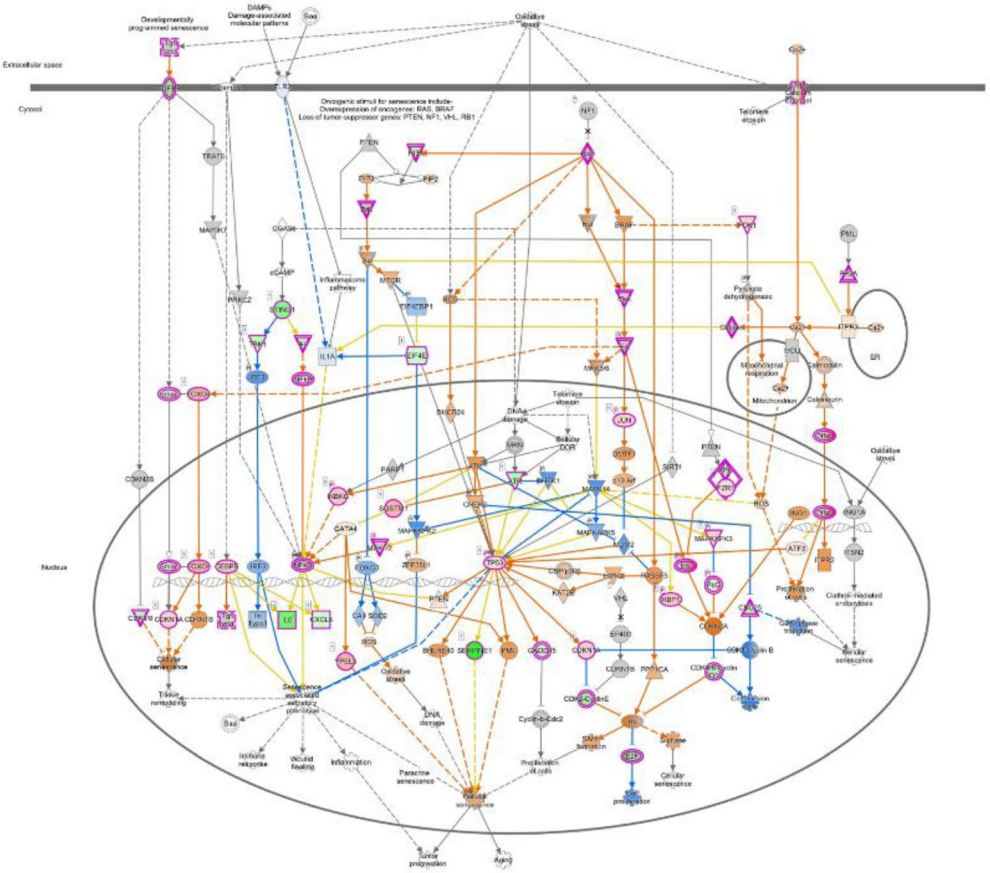
Sodium-glucose linked transporters (SGLTs) and facilitated diffusion glucose transporters (GLUTs) are two types of glucose transporters (Navale and Paranjape 2016)). GLUT proteins are thought to be mainly responsible for glucose transport. Interestingly, GLUT expression has been reported to be increased in DR patients, and suppression of GLUT1 expression helps alleviate the pathogenesis of DR (Lu et al. 2013). In our study, prolonged long-term high-glucose exposure caused a decrease in glucose uptake and attenuated proliferation (Fig. 1); therefore, we suggest that there may be other types of glucose transport that may be affected by prolonged high-glucose exposure and may cause aberrations in their expression or activities. For example, glucose SGLTs are reported to be expressed in retinal pericytes and mesangial cells (Wakisaka and Nagao 2017). In vitro, short-term exposure (three days) to high glucose levels has been reported to decrease the viability, wound healing capacity, and proliferation of RPE cells (Haranahalli Shivarudrappa et al. 2019).

Zhang et al. (2018, 2022) also discovered that after 24 h of high-glucose exposure, the expression of superoxide dismutase 2 (SOD2), a hallmark of mitochondrial redox homeostasis, and catalase in ARPE-19 cells was reduced and Shivarudrappa et al. also discovered decreased SOD2 activity in ARPE-19 cells cultured in high-glucose conditions for 24 and 48 h (Haranahalli Shivarudrappaet al. 2019; Shivarudrappa and Ponesakki 2020). Furthermore, hyperglycemia-induced upregulation of miR-34a has been reported to elevate ROS levels and mitochondrial dysfunction by inhibiting SIRT-1 and SOD2, causing impairment of microvascular dysfunction and the pathogenesis of DR (Scott et al. 2010; Zaidi et al. 2021). Although the above studies of high glucose exposure are short-term based, our results showed increasing ROS levels and a decreased level of SOD2 in long-term glucose-exposed ARPE-19 cells (Fig. 2), whereas the anti-proliferation was rescued following treatment with NAC, a ROS scavenger (NAC) (Fig. 3). Therefore, our results suggested that the imbalance of mitochondrial redox homeostasis could play an important role in high glucose-mediated pathophysiological degeneration of RPE cells.

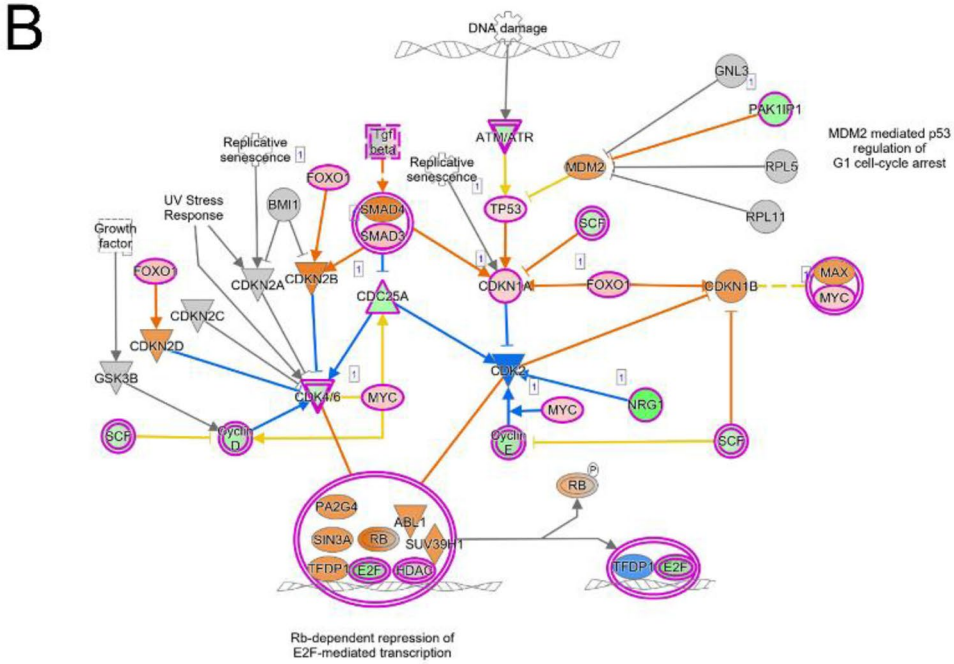
Mitochondrial superoxide may improve cellular function by stimulating the action of AMPK, which decreases glucose uptake and stimulates mitochondrial biogenesis by stimulating PGC1α (Kukidome et al. 2006; Mackenzie et al. 2013). AMPK has been shown to directly promote autophagy by phosphorylating autophagy-related proteins such as mTORC1, PIK3C3/VPS34 complexes, and Unc-51-like autophagy activating kinase (ULK1) or indirectly by tuning the levels of autophagy-associated transcription factors including BRD4, FOXO3 and TFEB (Li and Chen 2019). For example, metformin, a well-known drug used to treat diabetes, has been shown to enhance autophagic activity through AMPK phosphorylation and AMPK/mTOR signaling (Song et al. 2021; Zhao et al. 2020). In contrast, our results showed that long-term high-glucose exposure caused constitutive activation of AMPK by phosphorylation. It is speculated that under long-term high-glucose conditions, RPE cells try to upregulate AMPK phosphorylation to promote autophagy to overcome prolonged high-glucose-induced stresses. However, high glucose exposure may impair autophagic function (Fig. 4).

Mechanistically, the unfolded protein response modulates oxidative stress primarily via the PERK and activating transcription factor 6 (ATF6) signaling pathways (Rashid et al. 2015). In terms of autophagy, ATF4 and ATF6 can initiate the 5′ AMPK pathway, which is a critical regulator of energetic homeostasis in cells (Ai et al. 2020). Furthermore, excessive cytosolic calcium can contribute to autophagy modulation under ER stress through three signaling pathways: induction of CamKK/AMPK to suppress mTOR, stimulation of AMPK-mediated Beclin1 phosphorylation, and initiation of the PKC pathway (Dehdashtian et al. 2018; Hoyer-Hansen et al. 2007). The accumulation of damaged mitochondria is fueled by an age-dependent decrease in mitophagic flux, which fuels senescence induction by increasing ROS levels and other mediators. Previous studies have often used the results of short-term high-glucose studies to suggest that activation of the AMPK or AMPK-downstream pathway can reduce damage related to DR (Guo et al. 2020; Kim et al. 2018; Tang et al. 2022). Interestingly,

A



B


DNA damage
ATM/ATR
GNL3
PAK1IP1
RPL5
RPL11
MDM2
MDM2 mediated p53 regulation of G1 cell-cycle arrest
TP53
Tgf beta
Replicative senescence
Replicative senescence
FOXO1
BMI1
UV Stress Response
Growth factor
SMAD4
SMAD3
SCF
CDKN2A
CDKN2B
CDC25A
CDKN1A
FOXO1
CDKN1B
MAX
MYC
CDKN2C
CDKN2D
GSK3B
CDK4/6
MYC
CDK2
MYC
NRG1
SCF
Cyclin D
Cyclin E
SCF
RB
P
PA2G4
ABL1
SIN3A
RB
SUV39H1
TFDP1
E2F
HDAC
TFDP1
E2F
Rb-dependent repression of E2F-mediated transcription

◂**Fig. 7** KEGG pathway analysis of high glucose-initiated pathways in ARPE-19 cells. KEGG pathways related to **A** senescence and **B** cell cycle progression that involve regulators in ARPE-19 cells responding to high-glucose stress. Orange: increased/upregulated; Green: decreased/downregulated; pink: affected/modified

our study revealed that long-term high-glucose treatment causes AMPK activation as well as PERK and ATF6 activation via Bip/Grp78, resulting in increased ER stress (Fig. 4). In healthy cells, the level of p62, an autophagosome marker, is decreased during autophagic flux, the index of autophagic activity. However, in cells with diabetic retinopathy, an imbalance in autophagic flux occurs and blocks the fusion of autophagosomes and lysosomes (Aragones et al. 2020; Rosa et al. 2016), causing accumulation or no changes in p62 in cells. For example, in vivo studies showed that impaired autophagic flux caused no changes in p62 protein in several disease models, including spinocerebellar ataxia and cardiac hypertrophy (Simonson et al. 2017; Vig et al. 2009). In addition, a decrease in LAMP2 expression and the accumulation of lipids hinder autophagic function, which could be the cause of RPE cell injury or degeneration (Notomi et al. 2019). Consistently, our results showed that no changes in LAMP2, a decrease in p62 and the accumulation of lipofuscin in long high glucose-exposed RPE cells may be the cause of impaired autophagic flux (Fig. 4).

Regarding the pathogenesis and cell fate after high glucose treatment, the high glucose-induced degeneration of RPE cells may be caused by apoptosis and senescence. Cellular senescence is an irreversible phenotype of cell growth arrest that is accompanied by a flattened morphology and active but dysregulated metabolism (Blasiak et al. 2017). There have been many studies on the impact of high glucose exposure on RPE cells, and both apoptosis and senescence have been reported, which depend on the glucose concentration and the duration of high glucose exposure. For example, high-glucose exposure for 7 days induces apoptosis of RPE cells by activating MAPK and impairing VEGF pathways (Maugeri et al. 2021). Senescence can be induced not only by the erosion of the replicative potential (replicative senescence) but also by both extrinsic and intrinsic stresses (stress-induced premature senescence, SIPS), which are highly associated with progressive degenerative diseases (Gonzalez-Osuna et al. 2021; Lewinska and Wnuk 2017). Additionally, high glucose-induced ROS production leads to a decrease in cell viability and downregulates endogenous ROS scavengers, including glutathione peroxidase, superoxide dismutase, catalase, and glutathione. Furthermore, hyperglycemia-induced upregulation of miR-34a has been reported to elevate ROS levels and mitochondrial dysfunction by inhibiting SIRT-1 and SOD2, causing the impairment of microvascular dysfunction and the pathogenesis of DR (Scottet al. 2010; Zaidi et al. 2021). Moreover, hyperglycemia induced elevated ROS levels and mitochondrial function by downregulating the expression of SIRT-1 and SOD2 in mitochondria and SIPS induction, causing DR pathogenesis (Thounaojam et al. 2019).

P53R2/RRM2B, a ribonucleotide reductase-induced component, is mainly induced transcriptionally by the p53 protein upon DNA damage (Kimura et al. 2003; Tanaka et al. 2000). p53R2 forms an active complex with RRM1 in response to DNA damage, supplying deoxyribonucleoside triphosphates (dNTPs) for DNA repair (Kuo et al. 2012). Shosha et al. have shown that short-term exposure to high glucose levels can lead to premature senescence of retinal endothelial cells (Shosha et al. 2018; Thounaojam et al. 2019). Regarding the molecular pathways, hyperglycemia significantly increases the expression of senescence-related proteins, such as $P21^{cip1/waf1}$ and P53, along with cellular stress in diabetic mice compared with nondiabetic mice (Shosha et al. 2018). In this study, our results showed that long-term exposure to high glucose increased the expression of the two DNA damage markers γ-H2AX and p53R2, and significant upregulation of the senescence marker proteins P53 and P21 may contribute to premature senescence in ARPE-19 cells (Fig. 5). Furthermore, the senescence-associated secretory phenotype (SASP) exerted by senescent cells increased the secretion of matrix proteases MMP-1 and MMP-3 and MMP-10, the protease regulators PAIs, uPA and cathepsin B and inflammatory cytokines such as IL-6, a critical factor of SASP (Li et al. 2020), and our preliminary results showed a dramatic increase in IL-6 release in the medium of high glucose-exposed RPE cells (data not shown). In contrast, apoptotic cell death generally does not trigger inflammatory responses or release inflammatory substances (Priante et al. 2019; Rock and Kono 2008); therefore, it is suggested based on the results of our study that ARPE-19 cells exposed to long-term exposure to high glucose may be more susceptible to the phenotype of premature senescent cells.

Our next-generation sequencing (NGS) analysis revealed that prolonged high glucose exposure upregulated the RNA levels of senescence-associated genes in ARPE-19 cells (Fig. 6). Consistently, KEGG pathway prediction showed downregulation of cell cycle progression pathways leading to cell growth arrest in ARPE-19 cells (Fig. 7). In addition, the NGS analysis also revealed some interesting findings different from most studies using short-term exposure to high glucose. We summarized several specific pathways, including PEDF signaling (Kim et al. 2019), the WNT/$Ca2^{+}$ pathway (Benchoula et al. 2021), and 3-phosphoinositide degradation (Goncalves et al. 2018), which have not been discussed or investigated in short- or long-term high glucose treatment of retinal tissue/cell models. Downregulation of PEDF signaling may be associated with concomitant cell death of retinal tissues. The

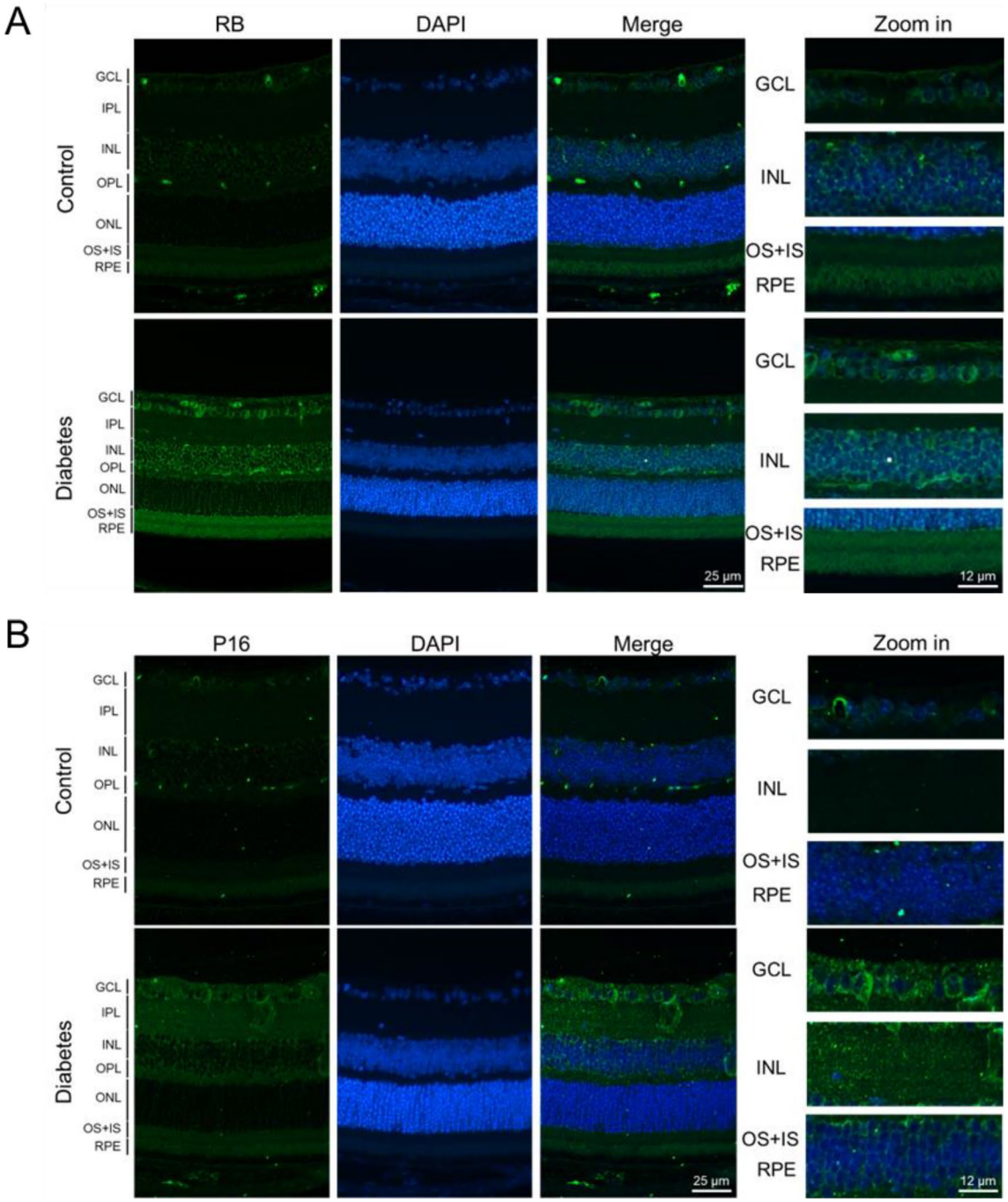


Fig. 8 The expression of senescence markers in the retinas of diabetic mice. **A** The representative results showed a significant increase in RB in the GCL, INL, OS + IS, and RPE layer of retinal tissue of diabetic mice. **B** The diabetic mice showed a significantly increased level of p16 in the GCL, IPL and INL layers of retinal tissue ($N=3$ in control mice, $N=3$ in diabetic mice). The retinal-related layers include the IPL (inner plexiform layer), INL (inner nuclear layer), ONL (outer nuclear layer), and RPE (retinal pigment epithelium) layers. For the original figures, the magnification is ×200, and the scale bar is 25 μm. For the zoom-in figures with a magnification of ×400. The scale bar for these zoom-in figures is 12.5 μm

downregulation of the WNT/$Ca2^+$ pathway may be related to the activated inflammatory mechanism. The activation of the 3-phosphoinositide-related pathway suggests the enhancement of the biosynthesis of 3-phosphoinositide and D-myo-inositol trisphosphate and leads to acceleration of the phosphoinositide 3-kinase central signal transduction axis controlling homeostasis and downstream regulator activation. Therefore, this is likely due to the

accumulation of intracellular stress responses in ARPE-19 cells under long-term high glucose incubation. In addition, IPA bioinformatics analysis revealed that more than 72 senescence-associated genes were activated in long-term high glucose -treated ARPE-19 cells. We believe the findings revealed deeper genetic and signaling modification by long-term high glucose stress exposure, which was a pattern more closely related to the effects of high glucose concentrations on ocular tissue in chronic diabetic patients.

Conclusions

In this study, our results suggested that long-term exposure to high glucose significantly increases intracellular ROS levels and ER stress and may cause impairment of autophagic function and premature senescence of the RPE, suggesting that it is involved in DR pathogenesis. Our study is the first to investigate the long-term effects of high glucose on RPE cells to simulate diabetes in diabetic patients, and it sheds light on the mechanism of RPE degradation during long-term high-glucose conditions. The established model and the results of this study should help clarify the pathophysiology of hyperglycemia-associated diseases and aid in the development of preventive drugs for diabetic eye diseases, including DR, in the future.

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Author Contributions C-CC and S-JS designed the experiments and wrote the paper. K-CC conceptualization. Y-HL, C-XH, and Y-DB performed the experiments and collected the data. Y-HL and Y-DB performed the bioinformatic analysis. C-CC and S-JS supply the funding acquisition. K-CC provided the cell line. K-CC, C-YL, C-YW, and H-MW provided reagents and translational suggestions. C-CC and S-JS wrote the draft. C-CC and S-JS checked and edited the manuscript. All authors approved the manuscript.

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Data availability The data that support the findings of this study are available on request from the corresponding author, Prof. Sheu, upon reasonable request.

Declarations

Conflict of Interest The authors report no conflicts of interest.

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