

LIN28A attenuates high glucose-induced retinal pigmented epithelium injury through activating SIRT1-dependent autophagy

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Abstract

• **AIM:** To evaluate the effects of LIN28A (human) on high glucose-induced retinal pigmented epithelium (RPE) cell injury and its possible mechanism.

• **METHODS:** Diabetic retinopathy model was generated following 48h of exposure to 30 mmol/L high glucose (HG) in ARPE-19 cells. Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot tested the expression of the corresponding genes and proteins. Cell viability as well as apoptosis was determined through cell counting kit-8 (CCK-8) and flow cytometry assays. Immunofluorescence assay was adopted to evaluate autophagy activity. Caspase 3 activity, oxidative stress markers, and cytokines were appraised adopting their commercial kits, respectively. Finally, ARPE-19 cells were preincubated with EX527, a Sirtuin 1 (SIRT1) inhibitor, prior to HG stimulation to validate the regulatory mechanism.

• **RESULTS:** LIN28A was downregulated in HG-challenged ARPE-19 cells. LIN28A overexpression greatly inhibited HG-induced ARPE-19 cell viability loss, apoptosis, oxidative damage as well as inflammatory response. Meanwhile, the repressed autophagy and SIRT1 in ARPE-19 cells challenged with HG were elevated after LIN28A overexpression. In addition, treatment of EX527 greatly inhibited the activated autophagy following LIN28A overexpression and partly abolished the protective role of LIN28A against HG-elicited apoptosis, oxidative damage as well as inflammation in ARPE-19 cells.

• **CONCLUSION:** LIN28A exerts a protective role against HG-elicited RPE oxidative damage, inflammation, as well as apoptosis *via* regulating SIRT1/autophagy.

• **KEYWORDS:** LIN28A; retinal pigmented epithelial cells; high glucose; Sirtuin 1; autophagy; oxidative stress

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INTRODUCTION

Diabetic retinopathy is a prevailing microvascular complication from diabetes mellitus and is a predominant contributor to visual loss as well as visual damage in working-aged people. Reports from the International Diabetes Federation disclosed that there were 463 million people diagnosed with diabetes mellitus in 2019, the number of which was projected up to be 700 million by 2045 around the globe^[1]. Approximately 30% of patients with diabetes mellitus will develop to diabetic retinopathy if not timely treated, and the number is estimated to achieve 550 million in 2030^[2-3]. Nowadays, diabetic retinopathy therapy chiefly focusing on the vascular lesions possesses restricted effectiveness^[4]. Thereafter, for the purpose of the discovery of potential therapeutic targets and drugs for diabetic retinopathy, a deep understanding of the pathological mechanism of diabetic retinopathy is prominent.

Hyperglycemia has been widely recognized as the dominant denominator throughout the pathophysiology of diabetic retinopathy, characterized by retinal vasculature, retinal inflammation and neovascularization^[5]. Based on researching the cellular and molecular pathology of diabetic retinopathy, vision dysfunction is closely implicated in the damage of the retinal pigmented epithelium (RPE), the main component of the outer blood-retina barrier^[6-7]. The hyperglycemia condition may also bring about oxidative damage, inflammatory response as well as apoptosis in RPE cells, causing severe damage and ultimately contributing to the pathogenesis of diabetic retinopathy^[8-9]. Therefore, the study of RPE cells is beneficial to understand diabetic retinopathy onset and course.

LIN28, a highly conserved RNA binding protein firstly identified in the nematodes *Caenorhabditis elegans*, is widely expressed in early developmental stages which organizes cell proliferation and differentiation in different tissues^[10]. LIN28 has two homologs, LIN28A and LIN28B, which have similar structural and functional characteristics. LIN28A has been discovered to regulate various cellular processes and is involved in multiple diseases, including osteoarthritis, renal fibrosis and cancers^[11-14]. Recent studies have shown that LIN28A exerts the therapeutic potential of enhancing glucose uptake and insulin sensitivity, thereby alleviating glucose metabolism-related diseases^[15-16]. It has been revealed that LIN28A obstructs the destruction of pancreatic β -cell death in diabetes, suggesting LIN28A as a critical regulator in the treatment of the diseases related to metabolism, diabetes is included^[17]. Upregulation of LIN28A has been reported to protect against hyperglycemia-induced injury in neonatal rat cardiomyocytes, thus retarding the development of diabetic cardiomyopathy^[18-19]. In addition, LIN28A has been confirmed to be an important regulator during zebrafish retina regeneration^[20]. The counteractive role of rasagiline combined with idebenone in ischemia-reperfusion-stimulated retinal injury was validated through enhancement of LIN28A/let-7, highlighting the importance of LIN28A mediating neuroprotection and restoring visual function^[21]. Although these observations suggest LIN28A has a prominent role in diabetes mellitus complications and retinal regeneration, the issue of whether LIN28A functions during diabetic retinopathy has yet not to be introduced.

The aim of the current study was to illustrate the specific role of LIN28A in hyperglycemia-mediated RPE damage and attempted to clarify its potential mechanism, providing a better understanding of the molecular targets for diabetic retinopathy therapy.

MATERIALS AND METHODS

Cell Treatment Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) as well as 1% penicillin-streptomycin was applied for the cultivation of ARPE-19 cell supplied by American Type Culture Collection (ATCC; Manassas, VA, USA) under a 5% CO₂ and 95% air atmosphere at 37°C.

ARPE-19 cells were stimulated by high glucose (HG; 30 mmol/L) for 48h to construct an *in vitro* diabetic retinopathy cell model. Meanwhile, cells were treated with normal glucose (NG; 5.5 mmol/L) as the control. In addition, ARPE-19 cells were pretreated with 10 μ mol/L of EX527 (Selleck Chemicals, Shanghai, China), a Sirtuin 1 (SIRT1) inhibitor, for 10min prior to exposure to HG.

Quantitative Real-Time Polymerase Chain Reaction After determination of the purity and concentration of the extracted RNA from ARPE-19 cells adopting Trizol (Invitrogen), the reverse transcription of complementary DNA (cDNA) from total 1 μ g of RNA was executed utilizing the M-MLA reverse transcription kit (Promega). Thereafter, with the application of LightCycler 480 SYBR Green I Master (Roche), the quantitative real-time polymerase chain reaction (qRT-PCR) experiment was carried out as per the manufacturer's recommended protocol. According to the $2^{-\Delta\Delta Ct}$ method, β -actin was selected for the calibration of gene expression.

Western Blot Total protein was prepared from ARPE-19 cells *via* trypsinization and radio immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis, MO, USA) lysis. After determination of protein concentration by bicinchoninic acid (BCA) assay (Solarbio, Beijing, China), the same amount of protein (30 μ g/lane) was fractionated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then shifted to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After being blocked by 5% skimmed milk for 1h, the membranes were labeled by LIN28A (ab124765, Abcam, USA), Bcl-2 (ab32124, Abcam), Bax (ab32503, Abcam), cleaved poly ADP-ribose polymerase (PARP1; ab32064, Abcam), PARP1 (ab227244, Abcam), SIRT1 (#2310, Cell Signaling Technology, USA), LC3B (ab192890, Abcam), Beclin-1 (ab207612, Abcam), p62 (ab109012, Abcam), and GAPDH (ab9485, Abcam) primary antibodies at 4°C overnight, followed by the supplementation of goat anti-rabbit IgG secondary antibody (ab6721, Abcam) for 2h of cultivation at room temperature. An enhanced chemiluminescence kit (Amersham, Piscataway, NJ, USA) was adopted for the development of immunoblots, which was subjected to quantification by Image J software (NIH, USA).

Cell Transfection The full length of human LIN28A cDNA was synthesized and subcloned in the pcDNA3.1 vector to generate LIN28A expression plasmid (Oe-LIN28A; Guangzhou RiboBio Co., Ltd., China), designating the blank pcDNA3.1 as Oe-NC. By virtue of Lipofectamine 3000 reagent (Invitrogen), Oe-LIN28A or Oe-NC was delivered into ARPE-19 cells upon 60% to 70% confluence as per the guidelines provided by the manufacturer. Totally 48h post transfection, the efficacy was determined by detecting the expression level of LIN28A.

Cell Counting Kit-8 Assay ARPE-19 cells were inoculated into the 96-well plates (5×10^2 cells/well) and subjected to indicated treatment in each group. Thereafter, each well was given 10 μ L of cell counting kit (CCK)-8 (Dojindo Molecular Technologies Inc., Kumamoto, Japan) for extra 2h of cultivation at 37°C. Under a microplate reader (Multiskan; Thermo Fisher Scientific), OD450 was monitored.

Flow Cytometry Analysis The Annexin V-FITC apoptosis kit (BD Biosciences, Franklin Lakes, NJ, USA) was applied for the measurement of cell apoptotic rate. Following the digestion with trypsin, washing with ice-cold phosphate-buffer-solution (PBS) and suspension in ice-cold PBS, ARPE-19 cells were dyed by 5 μ L Annexin V-FITC/prodium iodide (PI) away from light for 15min at 37°C. The FACS CantoII flow cytometry (BD Biosciences) was applied for the calculation of cell apoptotic rate.

Caspase 3 Activity Caspase 3 content was tested strictly in line with the manufacturer's guidelines *via* the caspase 3 Activity Assay Kit (Beyotime Biotechnology, Shanghai, China). In brief, the extracted protein was incubated with Ac-DEVD-pNA (2 mmol/L) in darkness at 37°C for 4h. Under a microplate reader, OD450 was monitored.

Measurement of Oxidative Stress Levels The intracellular reactive oxygen species (ROS) level was detected by the commercial kit (Sigma-Aldrich) based on the conversion of 2',7'-dichlorofluorescein diacetate into highly fluorescent 2',7'-dichlorofluorescein (DCF) strictly in accordance with the user's guidelines. The fluorescence intensity was imaged *via* a fluorescent microscope (Nikon Corporation, Tokyo, Japan). In addition, the supernatants from ARPE-19 cells were collected by centrifugation at 850 g at 4°C for 15min. Superoxide dismutase (SOD; A001-3), malondialdehyde (MDA; A003-1), and glutathione peroxidase (GSH-Px; A005-1) contents were separately appraised using the relevant kits (Nanjing Jiancheng Bio Institute) referring to the producer's instructions.

Enzyme-Linked Immunosorbent Assay In the cell supernatants, interleukin-6 (IL-6; ml028583), IL-1 β (ml058059), and tumor necrosis factor-alpha (TNF- α ; ml077385) concentrations were separately examined with relevant enzyme-linked immunosorbent (ELISA) kits (MLBio, Shanghai, China) complying with the procedures supplied by the manufacturer. Under a microplate reader, OD450 was monitored.

Immunofluorescence ARPE-19 cells were inoculated into 6-well plates (1×10^4 cells/well) and subjected to exposure to HG for 48h. After being washed with PBS, respective immobilization and permeation with 4% paraformaldehyde and 0.5% Triton X-100 at room temperature, the cells sealed by 3% BSA (Boster Bio, Wuhan, China) were labeled by anti-LC3B antibody (ab192890, Abcam) at 4°C overnight and the Alexa Flour 488-conjugated goat anti-rabbit antibody (ab150077, Abcam) in the dark at room temperature. DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) staining was conducted to stain cells for 3min in the dark. Eventually, *via* a fluorescent microscope, the images were photographed.

Statistical Analysis All results were depicted in the format of mean \pm standard deviation (SD). The statistical differences were analyzed *via* Student's *t*-test or one-way analysis of variance

(ANOVA) coupled with Tukey's post hoc test utilizing GraphPad Prism (GraphPad, San Diego, CA, USA). $P < 0.05$ were considered significant.

RESULTS

Effect of LIN28A on Cell Apoptosis in HG-triggered ARPE-19 Cells First, HG or NG was used to challenge ARPE-19 cells, respectively. HG treatment greatly inhibited LIN28A expression in ARPE-19 cells relative to the NG group (Figure 1A-1B). To unravel the regulatory role of LIN28A, ARPE-19 cells were transfected to overexpress LIN28A, which was successful based on the magnitude of the upregulation of LIN28A by transfection of Oe-LIN28A relative to the Oe-NC group (Figure 1C-1D). Thereafter, HG was used to challenge the transfected or un-transfected ARPE-19 cells. The following CCK-8 assay revealed that the HG stimulation caused a remarkable decrease of cell viability, while LIN28A overexpression partly abolished the reduction (Figure 1E). In addition, HG also led to a dramatically elevated cell apoptotic rate by flow cytometry analysis, which was partly alleviated by LIN28A overexpression (Figure 1F-1G), suggesting that LIN28A could inhibit HG-elicited ARPE-19 cell apoptosis, which was further confirmed by the repression of caspase 3 activity following LIN28A overexpression in ARPE-19 cells exposed to HG (Figure 1H). All in all, HG resulted in the descending Bcl-2 expression and ascending Bax and cleaved PARP expression, whereas LIN28A partly reversed these alternations (Figure 1I).

Effects of LIN28A on Oxidative Damage as well as Inflammation in HG-elicited ARPE-19 Cells To assess the impacts of LIN28A on HG-induced oxidative damage and inflammatory cytokines, we explored the alternations in ROS generation, MDA, SOD and GSH-Px contents, and cytokine production. Figure 2A-2D showed that HG resulted in a significant elevation of ROS generation and MDA content but a remarkable reduction in SOD and GSH-Px contents, which were then partly abolished by LIN28A overexpression, indicating that LIN28A exerted an anti-oxidative activity against HG-elicited ARPE-19 cell oxidative damage. Meanwhile, it was indicated from Figure 2E-2G that the elevated proinflammatory cytokines in ARPE-19 cells challenged with HG, including IL-6, IL-1 β , and TNF- α , were prominently reduced upon LIN28A overexpression. Hence, LIN28A was suggested to restrict HG-triggered ARPE-19 cell inflammation.

Effects of LIN28A on Autophagy and SIRT1 in HG-challenged ARPE-19 Cells Next, we further illuminated the regulatory mechanism of LIN28A in HG-induced retinal injury. The immunofluorescence images in Figure 3A disclosed HG weakened expression of LC3B, the classical marker of autophagy, which was then strengthened following LIN28A overexpression, indicating that LIN28A overexpression could

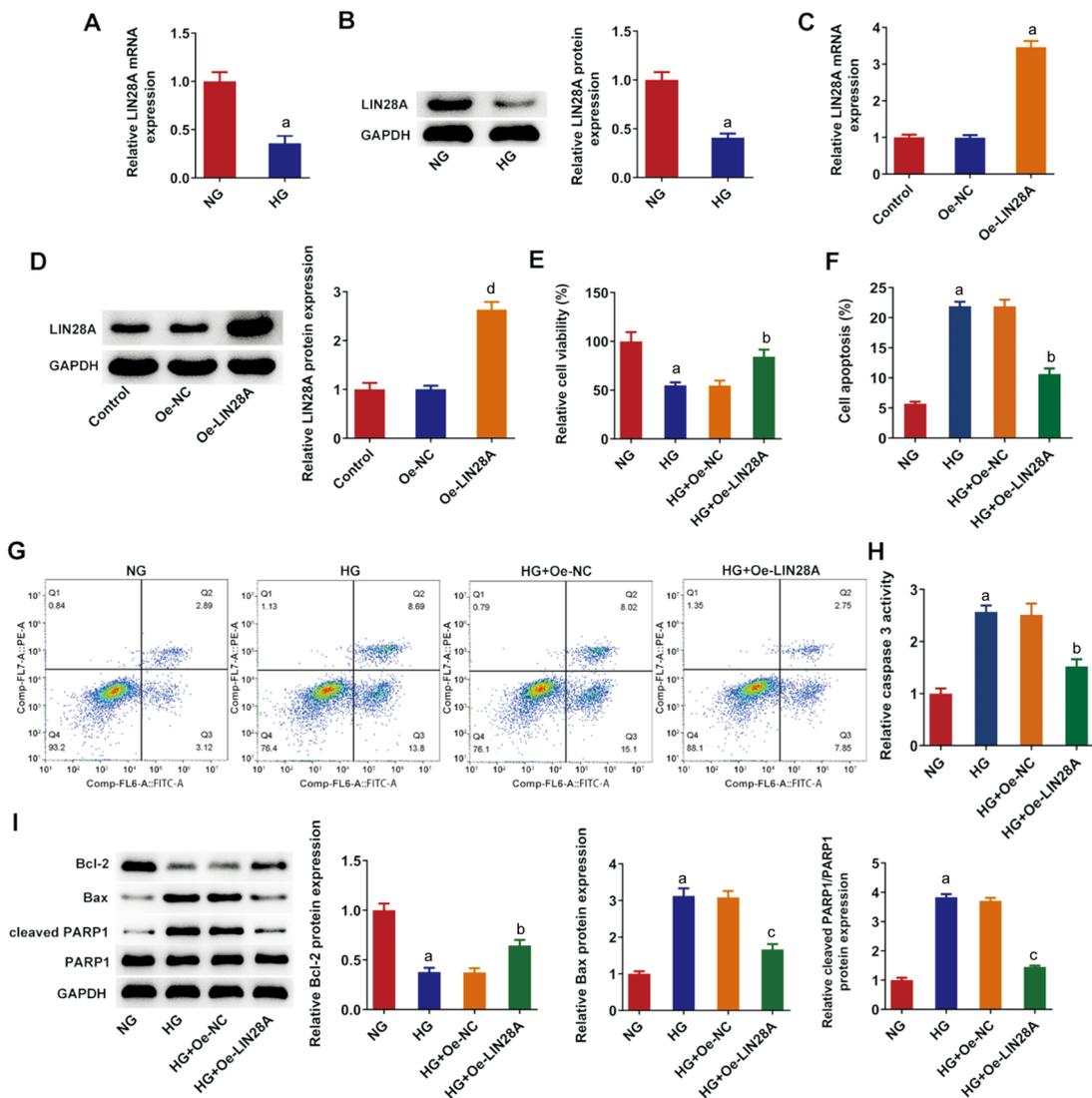


Figure 1 Effect of LIN28A on apoptosis in HG-triggered ARPE-19 cells. A: HG or NG was used to challenge ARPE-19 cells, respectively. qRT-PCR examined LIN28A mRNA level. B: Western blot examined LIN28A protein expression. C: After the delivery of oe-NC or oe-LIN28A into ARPE-19 cells, qRT-PCR examined LIN28A mRNA level. D: Western blot examined LIN28A protein expression. E: HG was used to challenge the transfected or un-transfected ARPE-19 cells. The CCK-8 method was used to analyze cell viability. F-G: Flow cytometry analysis was used to appraise cell apoptosis. H: Relevant kit was used test caspase 3 activity. I: Western blot was performed to examine Bcl-2, Bax, cleaved PARP1, PARP1 and GAPDH protein expression. ^a $P < 0.001$ vs NG; ^b $P < 0.01$ and ^c $P < 0.001$ vs HG+Oe-NC; ^d $P < 0.001$ vs Oe-NC. NG: Normal glucose; HG: High glucose; Oe-LIN28A: LIN28A overexpression vector; Oe-NC: Empty pcDNA3.1 vector.

activate autophagy in ARPE-19 cells challenged with HG. The subsequent Western blot exhibited that the downregulated LC3BII/I ratio as well as Beclin-1 expression and the raised p62 expression caused by HG exposure were partly retarded by LIN28A overexpression, further proving the role of LIN28A to activate HG-mediated autophagy (Figure 3B). In addition, activation of SIRT1 is one of the major signals that positively regulates autophagy. Accordingly, we also observed the inhibition of SIRT1 in ARPE-19 cells upon exposure to HG, which was then elevated on account of LIN28A overexpression, suggesting that the activated autophagy might be partly attributed to the upregulated SIRT1 following LIN28A overexpression.

Inhibitory Effect of SIRT1 on LIN28A-promoted Autophagy in ARPE-19 Cells Exposed to HG Subsequently, to identify whether SIRT1-mediated autophagy was responsible for the regulation of LIN28A during HG-mediated ARPE-19 cells, EX527, a SIRT1 inhibitor, was adopted to pretreat ARPE-19 cells before HG stimulation. As Figure 4A depicted, additional treatment of EX527 weakened the LC3B expression in comparison to HG+Oe-LIN28A group. Meanwhile, the promotive effects of LIN28A overexpression on LC3BII/I ratio and Beclin-1 expression and the counteractive role of LIN28A overexpression in p62 expression were weakened by the additional EX527 treatment (Figure 4B), indicating that LIN28A might activate autophagy *via* upregulating SIRT1.

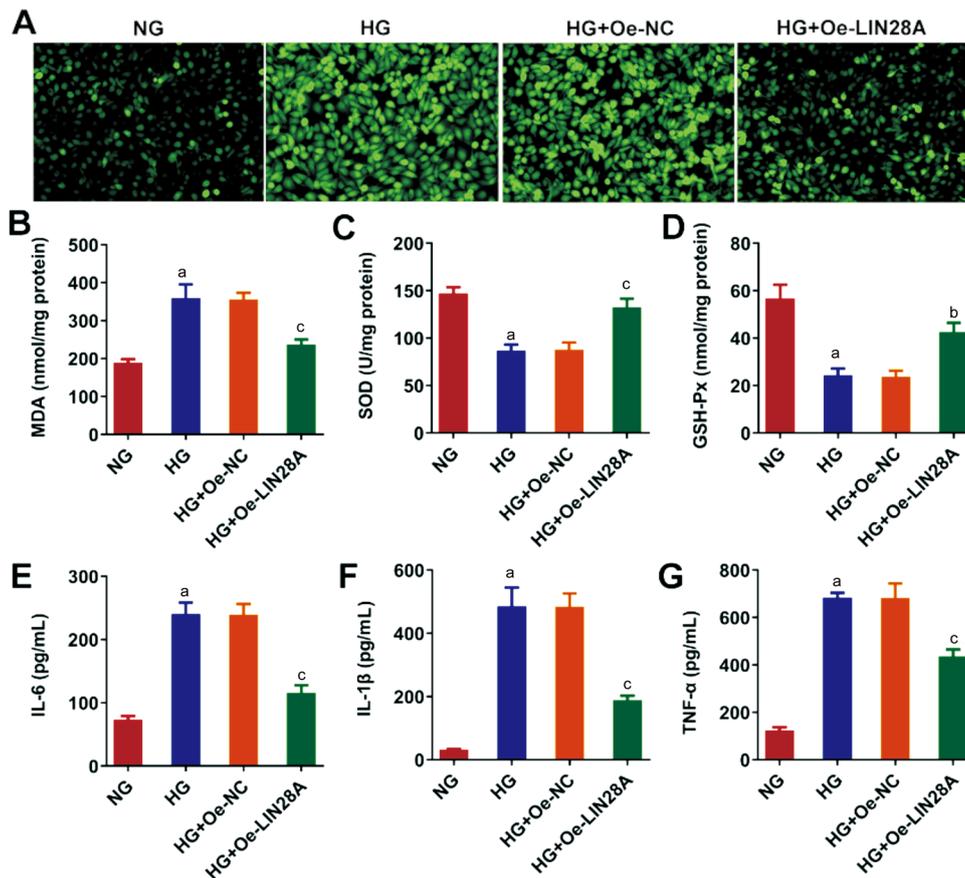


Figure 2 Effects of LIN28A on oxidative damage as well as inflammation in HG-elicited ARPE-19 cells. A: HG was used to challenge the transfected or un-transfected ARPE-19 cells. After 48h, culture supernatant was harvested. ROS production was evaluated by DCF method. Magnification $\times 200$. B-D: In the cell supernatants, relevant kits were used to examine SOD, MDA and GSH-Px contents. E-G: IL-6, IL-1 β , and TNF- α contents were subjected to ELISA assay for analysis. ^a $P < 0.001$ vs NG; ^b $P < 0.01$ and ^c $P < 0.001$ vs HG+Oe-NC. NG: Normal glucose; HG: High glucose; Oe-LIN28A: LIN28A overexpression vector; Oe-NC: Empty pcDNA3.1 vector; ROS: Reactive oxygen species; SOD: Superoxide dismutase; MDA: Malondialdehyde; GSH-Px: Glutathione peroxidase; IL-6: Interleukin-6; TNF- α : Tumor necrosis factor-alpha.

Inhibitory Effects of SIRT1 on LIN28A-reduced Apoptosis, Oxidative Damage, as well as Inflammation in HG-elicited ARPE-19 Cells Finally, we also evaluated the critical role of SIRT1 underlying the counteractive role of LIN28A in HG-triggered retinal injury. As presented in Figure 5A-5B, the suppressive role of LIN28A elevation in cell apoptotic rate and caspase 3 content in ARPE-19 cells challenged with HG were partly weakened due to additional EX527 treatment, which was consistent with the downregulated Bcl-2 and elevated Bax and cleaved PARP in HG+Oe-LIN28A+EX527 group compared to HG+Oe-LIN28A group (Figure 5C). Furthermore, the antioxidative activities of LIN28A in ARPE-19 cells exposed to HG were partially restricted on account of EX527, evidenced by the elevated ROS generation as well as MDA content and the reduced SOD and GSH-Px contents in HG+Oe-LIN28A+EX527 group compared with HG+Oe-LIN28A group (Figure 6A-6D). The inhibition on IL-6, IL-1 β , and TNF- α production imposed by LIN28A overexpression in ARPE-19 cells exposed to HG was hindered on account of additional administration with EX527 (Figure 6E-6G).

DISCUSSION

Diabetic retinopathy, one of the severe complications from diabetes mellitus, is a predominant contributor to visual loss worldwide. Although conventional clinical treatments are proven to be effective in alleviating diabetic retinopathy, this disease still remains incurable^[22]. Hence, it is a great importance to explore innovative strategies to improve diabetic retinopathy therapy. We have made the novel observation that LIN28A was aberrantly downregulated in ARPE-19 cells challenged with HG in the current work, and disclosed the therapeutic efficiency of LIN28A as a target for alleviating HG-induced oxidative stress, inflammatory response, and apoptosis in ARPE-19 cells. In addition, we verified that LIN28A played a protective role against HG-caused RPE injury through activating SIRT1/autophagy pathway. Thus, our results ought to prompt further study to determine if LIN28A upregulation provides a novel viable target for treating diabetic retinopathy.

Oxidative damage remains a crucial event in the pathogenesis of diabetic retinopathy. As the retina has a high glucose

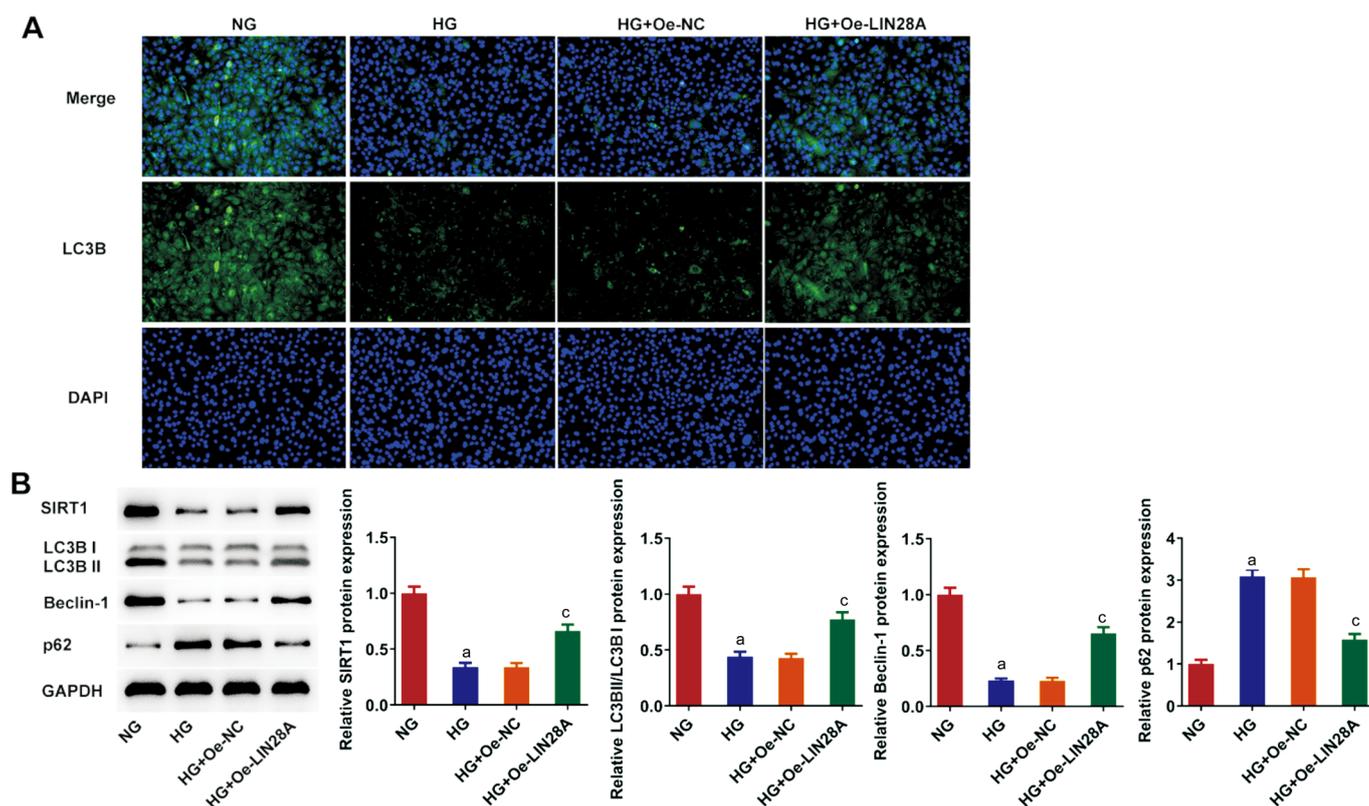


Figure 3 Effects of LIN28A on autophagy and SIRT1 in HG-challenged ARPE-19 cells A: HG was used to challenge the transfected or untransfected ARPE-19 cells. Cell immunofluorescence assay was performed to assess LC3B. Magnification $\times 200$. B: Western blot was conducted to test SIRT1, LC3B II/I, Beclin-1, p62 and GAPDH protein expression. ^a $P < 0.001$ vs NG; ^c $P < 0.001$ vs HG+Oe-NC. NG: Normal glucose; HG: High glucose; Oe-LIN28A: LIN28A overexpression vector; Oe-NC: Empty pcDNA3.1 vector; SIRT1: Sirtuin 1.

oxygen uptake reaction, RPE cells in patients with diabetic mellitus are thus vulnerable to oxidative damage caused by hyperglycemia^[23-24]. Subsequently, oxidative stress triggers a series of cellular responses, including inflammatory response and cell apoptosis, accompanied with damaging cellular functions, contributing to the pathogenesis of diabetic retinopathy and retinal damage. Mounting evidence indicates that limiting oxidative and inflammatory responses, as well as cell apoptosis, can be highly helpful to prevent or attenuate diabetic retinopathy. For instance, Sun *et al*^[25] revealed a beneficial effect of nesfatin-1 in ARPE-19 cells exposed to HG *via* mitigating oxidative stress, inflammation and apoptosis; Hao and Gao^[26] indicated diosgenin as a prospective drug for diabetic retinopathy therapy, and diosgenin activated the AMPK/Nrf2/HO-1 signaling to effectively ease apoptosis, inflammation as well as oxidative damage in ARPE-19 cells challenged with HG. Focusing on LIN28A, the anti-oxidative and anti-inflammatory activity of LIN28A have been revealed by a previous study that LIN28A was able to alleviate inflammatory injury and oxidative stress in lipopolysaccharide-treated human periodontal ligament stem cells^[27]. Upregulation of LIN28A was also confirmed to be an effective target to suppress neuroinflammation and apoptosis to attenuate ischemic stroke-associated brain injury^[28]. Thus,

LIN28A possesses anti-inflammatory, anti-oxidative, and anti-apoptotic activities, to exert a promising therapeutic target for numerous diseases. Consistent with previous documents, the protective role of LIN28A against diabetic retinopathy was first revealed in this study, and LIN28A could protect against HG-mediated ARPE-19 cell oxidative damage, inflammation as well as apoptosis, suggesting LIN28A as a novel therapeutic target for diabetic retinopathy.

Autophagy is a highly conserved intracellular degradation process that eliminates damaged organelles and abnormal proteins. In addition to serving as an adaptive response to cellular stresses, autophagy has been also regarded as the major factor involved in maintaining homeostasis and normal cellular functioning of organelles, and autophagy disorder has been confirmed to contribute to the initiation and course of diabetes mellitus as well as its complications, such as diabetic nephropathy, diabetic cardiomyopathy and diabetic peripheral neuropathy^[29-32]. In recent years, the role of autophagy in diabetic retinopathy has been gradually uncovered. It has been revealed that overstimulation of autophagy is positively correlated with aggravated retinal cell death in animal models of diabetic retinopathy, accompanied with the upregulated Beclin-1 and LC3 II^[33]. Consistently, *in vitro* models of diabetic retinopathy, HG has been reported to elevate the number of

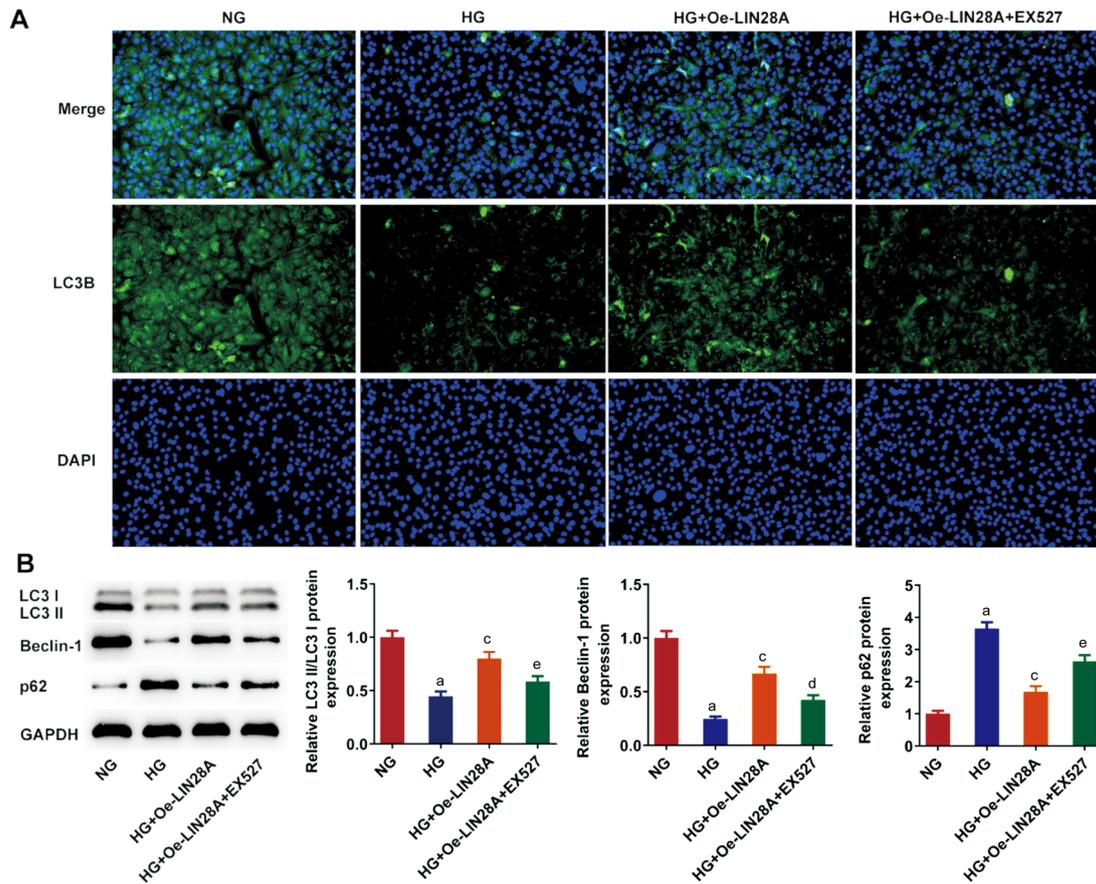


Figure 4 Inhibitory effect of SIRT1 on LIN28A-promoted autophagy in ARPE-19 cells exposed to HG A: Prior to exposure to HG, 10 μ mol/L of EX527, the SIRT1 inhibitor was used to preincubate ARPE-19 cells for 10min. Cell immunofluorescence assay was performed to assess LC3B. Magnification $\times 200$. B: Western blot was performed to examine SIRT1, LC3B I/II, Beclin-1, p62 and GAPDH protein expression. ^a $P < 0.001$ vs NG; ^c $P < 0.001$ vs HG; ^d $P < 0.01$ and ^e $P < 0.001$ vs HG+Oe-LIN28A. NG: Normal glucose; HG: High glucose; Oe-LIN28A: LIN28A overexpression vector; Oe-NC: Empty pcDNA3.1 vector.

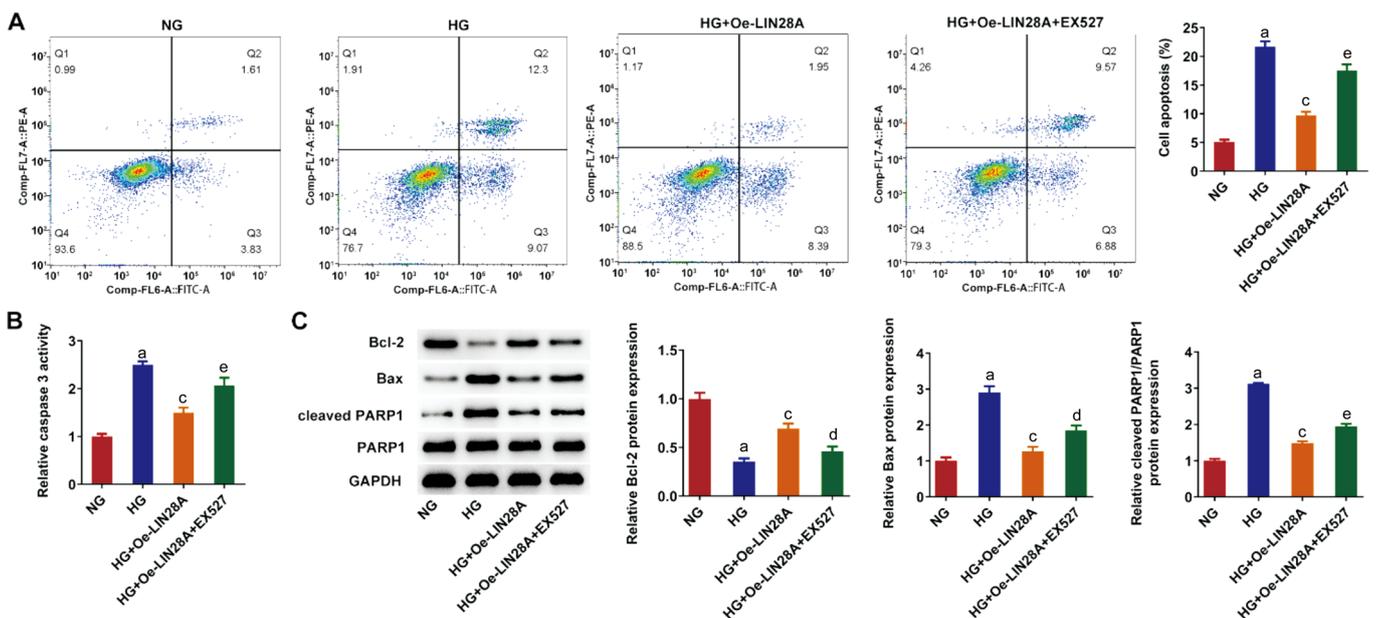


Figure 5 Inhibitory effect of SIRT1 on LIN28A-reduced apoptosis in HG-elicited ARPE-19 cells A: Prior to exposure to HG, 10 μ mol/L of EX527, the SIRT1 inhibitor was used to preincubate ARPE-19 cells for 10min. Flow cytometry analysis was conducted to appraise cell apoptosis. B: Relevant kit was used to test Caspase 3 activity. C: Western blot was used to examine Bcl-2, Bax, cleaved PARP1, PARP1 and GAPDH protein expression. ^a $P < 0.001$ vs NG; ^c $P < 0.001$ vs HG; ^d $P < 0.01$ and ^e $P < 0.001$ vs HG+Oe-LIN28A. NG: Normal glucose; HG: High glucose; Oe-LIN28A: LIN28A overexpression vector; Oe-NC: Empty pcDNA3.1 vector.

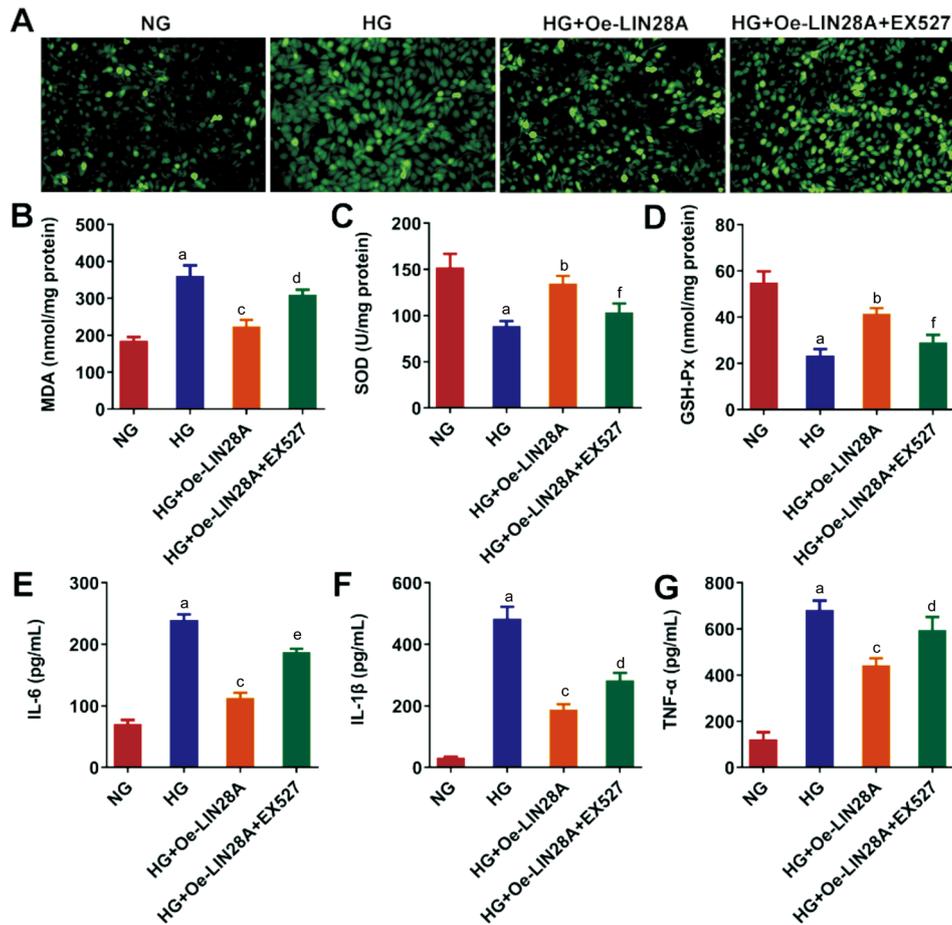


Figure 6 Inhibitory effect of SIRT1 on LIN28A-reduced oxidative stress and inflammation in HG-elicited ARPE-19 cells A: Prior to exposure to HG, 10 μmol/L of EX527, the SIRT1 inhibitor was used to preincubate ARPE-19 cells for 10min. Totally 48h post HG stimulation, culture supernatant was harvested. ROS production was evaluated by DCF method. Magnification ×200. B-D: In the cell supernatants, relevant kits were used to examine SOD, MDA and GSH-Px contents. E-G: IL-6, IL-1β and TNF-α contents were subjected to ELISA assay for analysis. ^a*P*<0.001 vs NG; ^b*P*<0.01 and ^c*P*<0.001 vs HG; ^d*P*<0.05, ^e*P*<0.01, and ^f*P*<0.001 vs HG+Oe-LIN28A. NG: Normal glucose; HG: High glucose; Oe-LIN28A: LIN28A overexpression vector; Oe-NC: Empty pcDNA3.1 vector; ROS: Reactive oxygen species; SOD: Superoxide dismutase; MDA: Malondialdehyde; GSH-Px: Glutathione peroxidase; IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-alpha; SIRT1: Sirtuin 1.

autophagosomes in ARPE-19 cells, and autophagy inhibitor 3-methyladenine was demonstrated to exert a protective effect on HG-challenged ARPE-19 cells^[34-35]. On the contrary, another *in vitro* study noticed remarkably reduced LC3B-II/I ratio and accumulated p62 in HG-induced RPE cells, and the autophagy inhibitors further aggravated HG-mediated injury in RPE cells^[36]. In addition, the latest study disclosed that HG repressed autophagy as well as obstructed autophagy degradation on account of the permeabilization of lysosome membrane in RPE cells, while knockdown of HMGB1 could exacerbate autophagy degradation, thereby protecting against inflammation and apoptosis in hyperglycemia-mediated RPE cells^[37]. In the present study, we also observed a weakened autophagy in ARPE-19 cells challenged with HG, evidenced by reduced LC3B and Beclin-1 and elevated p62 expression, while LIN28A could partly restore autophagy. Given the importance of autophagy in diabetic retinopathy, the protective role of LIN28A against hyperglycemia-mediated RPE

cell injury might be partially attributed to its regulation on autophagy.

SIRT1, an NAD⁺-dependent multifunctional enzyme, is a critical modulator of the autophagy process from initiation to degradation^[38]. Accumulating evidence reveals that SIRT1 serves an important role in ocular diseases through regulating diversified physiological and pathological processes, including oxidative stress, apoptosis as well as inflammation^[39-40]. It is well documented that SIRT1 was suppressed in the retinal tissues of rats with diabetic retinopathy, and SIRT1 activation can protect the retina from diabetes-mediated damage *via* diminishing oxidative stress and inflammation^[40-41]. In addition, artesunate can alleviate oxidative damage as well as inflammation in diabetic retinopathy *via* activating AMPK/SIRT1-regulated autophagy^[42]. Arbutin is verified to play a protective role against diabetic retinopathy through inhibiting HG-mediated inflammation and apoptosis in ARPE cells by promoting SIRT1-dependent autophagy^[43]. Therefore, SIRT1

signaling and SIRT1-dependent autophagy are crucial to mediate oxidative stress and inflammation during diabetic retinopathy, and the therapeutic potential of strategies targeting SIRT1 activation has been acknowledged to ameliorate the progression of diabetic retinopathy^[44-45]. Consistently, in this study, it was observed that the repressed SIRT1 in ARPE-19 cells challenged with HG was greatly augmented following LIN28A overexpression. Furthermore, treatment of SIRT1 inhibitor EX527 partially hindered the counteractive role of LIN28A in HG-elicited ARPE-19 cell inflammation, oxidative damage as well as apoptosis. Meanwhile, the promoted autophagy by LIN28A was also partly repressed by EX527. The above data suggested that LIN28A might ameliorate HG-triggered ARPE-19 cell oxidative damage, inflammation as well as apoptosis through activating SIRT1-mediated autophagy. Considering the critical role of SIRT1 in influencing diabetic retinopathy as aforementioned, our study introduced the therapeutic potential of LIN28A targeting SIRT1 activating to affect the development of diabetic retinopathy.

However, limitations still exist in this study. This study is concentrated on the role and regulation of LIN28A in an *in vitro* diabetic retinopathy cell model. The *in vivo* animal model and the analysis of clinical samples will be conducive to the validation of these findings, which are now planned for future work.

Taken together, this work disclosed the antioxidative, anti-inflammatory, as well as antiapoptotic effects of LIN28A on hyperglycemia-mediated RPE cell injury. The beneficial role of LIN28A in ARPE-19 cells challenged with HG may be achieved partly through activating SIRT1/autophagy pathway. Our results here provided a novel role for LIN28A in regulating ARPE-19 cells challenged by HG and offered a possible therapeutic target for diabetic retinopathy.

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