



OPEN ANXA2 regulates mitochondrial function and cellular senescence of PDLCs via AKT/eNOS signaling pathway under high glucose conditions

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Diabetes mellitus is one of the risk factors for periodontitis. Patients with diabetes mellitus possess higher prevalence of periodontitis, more severe periodontal destruction, yet the underlying mechanisms of action are not yet clear. Annexin A2 (ANXA2) is a calcium-dependent phospholipid-binding protein widely involved in membrane repair, cytokinesis, and endocytosis. In this study, we explore whether ANXA2 is one of the associative links between diabetes and periodontitis and find out its underlying mechanisms. Cellular senescence and mitochondrial functions (ROS, mitochondrial morphology, mitochondrial autophagy) were observed. We observed that ANXA2 expression was down-regulated in Periodontal ligament cells (PDLs) under high glucose conditions. Furthermore, overexpression of ANXA2 delayed high glucose-induced cellular senescence and mitochondrial dysfunction. β -galactosidase activity and the mRNA levels of the senescence-related genes (*p21*, *p16*) were decreased, mitochondrial fracture and ROS release were reduced, and the expression of mitochondrial autophagy-related proteins (LC3, p62, Parkin) was enhanced. Mechanistically, we demonstrated that it can regulate the AKT/eNOS signaling pathway by knockdown and overexpression of ANXA2 which was measured using Western blotting (WB) assay to measure the expression of eNOS, p-eNOS Ser1177, Akt and p-Akt Ser473 proteins in PDLs. After that, we used AKT and eNOS inhibitors to demonstrate the protective effect of ANXA2 on PDLs under high glucose conditions. The above results suggest that ANXA2 has an anti-aging protective effect, attenuates high glucose-induced cellular senescence in PDLs, and maintains mitochondrial homeostasis. Therefore, it would be valuable to further explore its role in the link between diabetes and periodontitis in future experiments.

Keywords Mitochondria, Cellular senescence, Mitophagy, Diabetes, Periodontitis

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia¹. The World Health Organization (WHO) estimated that 42.28 billion adults worldwide had diabetes mellitus in 2014, a number that has continued to grow rapidly in recent years². Periodontitis is the sixth complication of diabetes mellitus³. It is manifested by the fact that patients with diabetes mellitus possess higher prevalence of periodontitis, more severe periodontal destruction, and a faster rate of progression⁴. The prevalence of periodontitis in patients with diabetes mellitus has been increasing over the past decades, and the number of patients with diabetes mellitus is increasing rapidly in the past few years. For decades, a large number of studies have searched for mechanisms of action to better control severe periodontitis in diabetic patients. However, to date, the underlying molecular mechanisms remain unclear.

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Senescence is a well-recognized risk factor for periodontitis⁵ and hyperglycemia-induced cellular senescence exacerbates periodontal lesions⁶. During the aging process, cellular senescence undergoes a phase of early senescence response followed by a senescence-associated secretory phenotypic (SASP) response^{7,8}. The heavier burden of senescent cells and SASP in diabetes can lead to tissue destruction^{9,10}, including inflammatory bone loss in periodontitis.

Annexin A2 (ANXA2) is a calcium-dependent phospholipid-binding protein widely involved in membrane repair, cytokinesis, and endocytosis¹¹. It is widely expressed in endothelial cells, monocytes, cancer cells, and osteoblasts¹². It has been demonstrated that the expression of ANXA2 is significantly reduced in patients with diabetes mellitus¹³. In addition, ANXA2 has been found to be an early glycosylation product in experimental diabetes mellitus¹⁴. It has been reported that sustained hyperglycemia (7 days) induces reduced expression of ANXA2 in human brain microvascular endothelial cells and causes dysfunction¹⁵. It then appears to be of great interest whether the reduction of ANXA2 is one of the reasons for the worsening of periodontitis in diabetic patients.

To investigate whether ANXA2 is one of the associative links between diabetes and periodontitis, our study explored the changes in ANXA2 expression in Periodontal ligament cells (PDLs) under high glucose condition, confirming that ANXA2 regulates mitochondrial function and cellular senescence in PDLs as well as its underlying mechanisms. Overall, our findings suggest that ANXA2 is a key target for PDLs to resist high glucose-induced cellular damage. Therefore, it would be valuable to further explore its role in the link between diabetes and periodontitis in future experiments.

Materials and methods

Ethics statement

Informed consent must have been obtained from a parent and/or legal guardian, and ethical approval for the study was granted by the Ethics Committee of the Affiliated Hospital of Hangzhou Normal University, with the reference number 2023(E2)-KS-034. Research involving human research participants must have been performed in accordance with the Declaration of Helsinki. All experiments were performed in accordance with relevant guidelines and regulations.

Isolation and culture of PDLs

A total of thirty intact third or premolar teeth, free from periodontal disease or dental caries, were collected from volunteers aged between 10 and 25 years who were undergoing extraction for orthodontic purposes.

The process for isolating PDLs entailed gently removing the periodontal ligament from the root's midsection and treating it with 2 mg/ml of collagenase type I (Biosharp, Heifei, China) at a temperature of 37 °C for a duration of 30 min. Subsequently, the cells were meticulously placed into Dulbecco's modified Eagle medium (DMEM; Cellmax, China), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Solarbio, Beijing, China). The PDLs were then cultured at 37 °C in a humidified atmosphere with 5% CO₂, with the culture medium being refreshed every three days. Upon reaching approximately 80% confluence, the cells were treated with 0.25% trypsin-EDTA (HyCyt, Suzhou, China) for digestion and subsequent subculturing. For this research, cells from passages P3 to P6 were selected.

Small interfering RNAs (siRNAs) and plasmid DNA transfection

Knockdown of ANXA2 #1, #2, #3 were performed with siRNA targeting ANXA2, 5'-GUCUGUCAAAGCC UAUACUTT-3', 5'-UUGCUGAUCGGCUGUAUGATT-3', 5'-ACCAACCGCAGCAAUGCACTT-3'. The pcDNA3.1-ANXA2 plasmid was obtained from GenePharma, China. Follow the manufacturer's instructions for Lipofectamine 2000 (Invitrogen, 11668019) for transfection procedures. Before transfection, incubate the mixture (OPTI-MEM medium, siRNA or plasmid, Lipofectamine 2000) at room temperature for 15 min, then co-incubate it with PDLs for 6 h to complete the transfection process. After incubation, replace the existing medium with basal medium to terminate the transfection reaction. The cells can be used for subsequent experimental steps 48 h post-transfection.

Senescence-associated β -galactosidase (SA- β -Gal) staining

The SA- β -Gal Staining technique was utilized to assess the senescence of PDLs due to exposure to high glucose¹⁶. Following a 2-day incubation with high glucose, the SA- β -gal activity in PDLs was measured in accordance with the instructions provided by the manufacturer (Solarbio, China). The culture medium was removed, and the wells were rinsed twice with PBS before being fixed with 4% paraformaldehyde (PFA) for 5 min at ambient temperature. After PFA removal, 2 ml of SA- β -Gal staining solution was added to each well, and the staining process was carried out at 37 °C for a duration of 24 h. The aged PDLs exhibited a blue coloration, which was visible under an inverted microscope (Nikon, Japan). The comparative intensity of the SA- β -gal staining was quantified using Image J software for analysis.

Observation of intracellular ROS

The ROS Assay Kit (Solarbio, China) was employed to measure the levels of intracellular reactive oxygen species (ROS). PDLs were grown in a high-glucose medium for a period of 8 h. Subsequently, the cells were treated with 200 µl of a 0.1% solution of dihydroethidium (DHE) in DMEM and maintained at 37 °C for 20 min. Following this, the cells were rinsed three times with phosphate-buffered saline (PBS), and the presence of intracellular ROS was examined using a confocal microscope (OLYMPUS, Tokyo, Japan). Images were then analyzed using Image J software for quantification.

Mito-tracker

Mito-Tracker (Beyotime, China) is used to observe the morphology of mitochondria in cells. When the cells were cultured in a high glucose environment for 2 days, we observed the morphology of their mitochondria. According to the product instructions, remove the cell culture medium, add Mito-Tracker Red CMXRos working solution, and incubate at 37 °C for 30 min. Remove the Mito-Tracker Red CMXRos working solution and add fresh cell culture solution pre-warmed and incubated at 37 °C. Laser confocal microscopy (OLYMPUS, Tokyo, Japan) was used for observation.

Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was employed to evaluate the impact of elevated glucose on the mRNA expression of senescence-associated genes (*p21*, *p16*) in PDLs. The cells were lysed and their total RNA was extracted using TRIzol reagent (Accurate Biology). RNA purification was conducted with the SteadyPure RNA Kit (Accurate Biology), adhering to the manufacturer's guidelines. A quantity of 1 µg of the isolated RNA was used as a template for cDNA synthesis, which was carried out using the 5× PrimeScript RT Master Mix (Accurate Biology), following the provided protocol. For the qPCR analysis, 2 µl of the synthesized cDNA was used in a 20 µl reaction mix with the SYBR Green Pro Taq HS qPCR Kit (Accurate Biology). The expression levels of the target genes, as listed in Table 1, were normalized against the internal control gene GAPDH. The relative quantification of gene expression was calculated using the 2^{-ΔΔCt} method.

Western blot analysis

Western Blot Analysis was performed as in previous experiment¹⁶. Proteins from PDLs were extracted using RIPA buffer (EpiZyme, China), and protein concentrations were quantified with a BCA Kit (EpiZyme, China). Each protein sample, amounting to 10 µg, was subjected to SDS-PAGE and then electroblotted onto PVDF membranes. These membranes underwent a blocking step with BSA (EpiZyme, China) for 15 min before being exposed to the primary antibody at 4 °C for an extended period. Post-washing with TBST, the membranes were treated with secondary antibodies at room temperature for a duration of 2 h. The antibodies were used as follows: ANXA2 (ab178677; 1:1000 diluted; Abcam, Cambridge, UK), p62 (ab109012; 1:10000 diluted; Abcam), LC3-B (ab192890;1:2000diluted; Abcam, USA), p-AKT(Ser473)(#9271;1:1000 diluted; CST, USA), AKT (#9272;1:1000 diluted; CST, USA), p-eNOS(Ser1177)(#9570;1:1000 diluted; CST, USA), eNOS (#9572;1:1000 diluted; CST, USA), Parkin (#2132, 1:1000 diluted; CST, USA), β-actin (380624; 1:5,000 diluted; Zenbio, Chengdu, China), goat anti-rabbit IgG (#511203; 1:5000 diluted; Zenbio, China). The presence of bands was revealed using an electrochemiluminescence detection reagent (Bio-Rad, Hercules, CA, USA). The intensity of these bands was analyzed and quantified using ImageJ software.

Immunofluorescence staining

Immunofluorescence staining was performed as in previous experiment¹⁷. PDLs were inoculated on coverslips of 12-well plates and fixed with 4% paraformaldehyde (PFA) for 20 min and permeabilized with 0.4% Triton X-100 for 20 min after 48 h under high glucose conditions. The cells were then incubated with BSA (EpiZyme, China) for 15 min at room temperature. Cells were incubated with primary antibody ANXA2 (ab178677; 1:250 diluted; Abcam, Cambridge, UK), overnight at 4 °C, rinsed with PBS, and incubated at room temperature with secondary antibody (#511201, 1:100 diluted, Zenbio, China) for 1 h. Hoechst 33,342 (Thermo Fisher Scientific) was used for nuclear DNA staining. Laser confocal microscopy (OLYMPUS, Tokyo, Japan) was used for observation.

Statistical analysis

The statistical analysis of all data was performed using GraphPad Prism software (version 6.0, USA), with results depicted as the mean ± SEM based on three individual experiments. Variability among the groups was assessed through one-way ANOVA and the differences between two groups were analyzed using Student's t-test. Data were deemed to have statistical significance if the *P* < 0.05.

Result

ANXA2 expression is suppressed in PDLs under high glucose condition

In order to test whether the expression of ANXA2 is altered in human PDLs under high glucose conditions, we examined the expression level of ANXA2 by western blotting in both control (CTRL) and high glucose groups(40G) at 48 h, and found that its expression was suppressed in PDLs under high glucose conditions

Target Gene		Nucleotide Sequence
p16	Forward	GGGTCGGGTAGAGGAGGTG
	Reverse	CCATCATCATGACCTGGATCGG
p21	Forward	TCACTGTCTTGTACCCTTGTGCCTC
	Reverse	GGCGTTTGGAGTGGTAGAAATCTGTC
GAPDH	Forward	GGACTCATGACCACAGTCCA
	Reverse	TCAGCTCAGGGATGACCTTG

Table 1. Primer.

(Fig. 1A,B). Furthermore, high glucose conditions reduced ANXA2 expression in the cytoplasm of PDLCS (Fig. 1C,D).

ANXA2 attenuates cellular senescence of PDLCS in high glucose condition

Numerous studies have shown that high glucose induces cellular senescence^{18,19}. Our preliminary experiments¹⁶ revealed that when PDLCS were cultured under high-glucose conditions (40 mM) for 24 h, their proliferative activity was inhibited, thereby ruling out the possibility of cellular senescence induced by high confluence. We speculated that cellular senescence caused by high glucose was related to the suppression of ANXA2 expression. To verify our hypothesis, we cultured ANXA2 overexpressing cells in a high glucose environment (CTRL: control; 40G: 40mM glucose; 40G-A: 40mM + ANXA2 overexpression). 48 h later, Senescence-Associated β -Galactosidase (SA- β -Gal) Staining was performed, and it was found that the SA- β -gal activity in the high glucose group was significantly higher compared with that in the control group. Meanwhile, SA- β -gal activity was attenuated in 40G-A compared to 40G (Fig. 2A-B). In addition, qPCR detection of cellular senescence-related genes showed that the expression of *p21*, *p16* was also significantly decreased in the 40G-A compared with 40G (Fig. 2C-D). This indicated that ANXA2 could inhibit cellular senescence of PDLCS caused by high glucose.

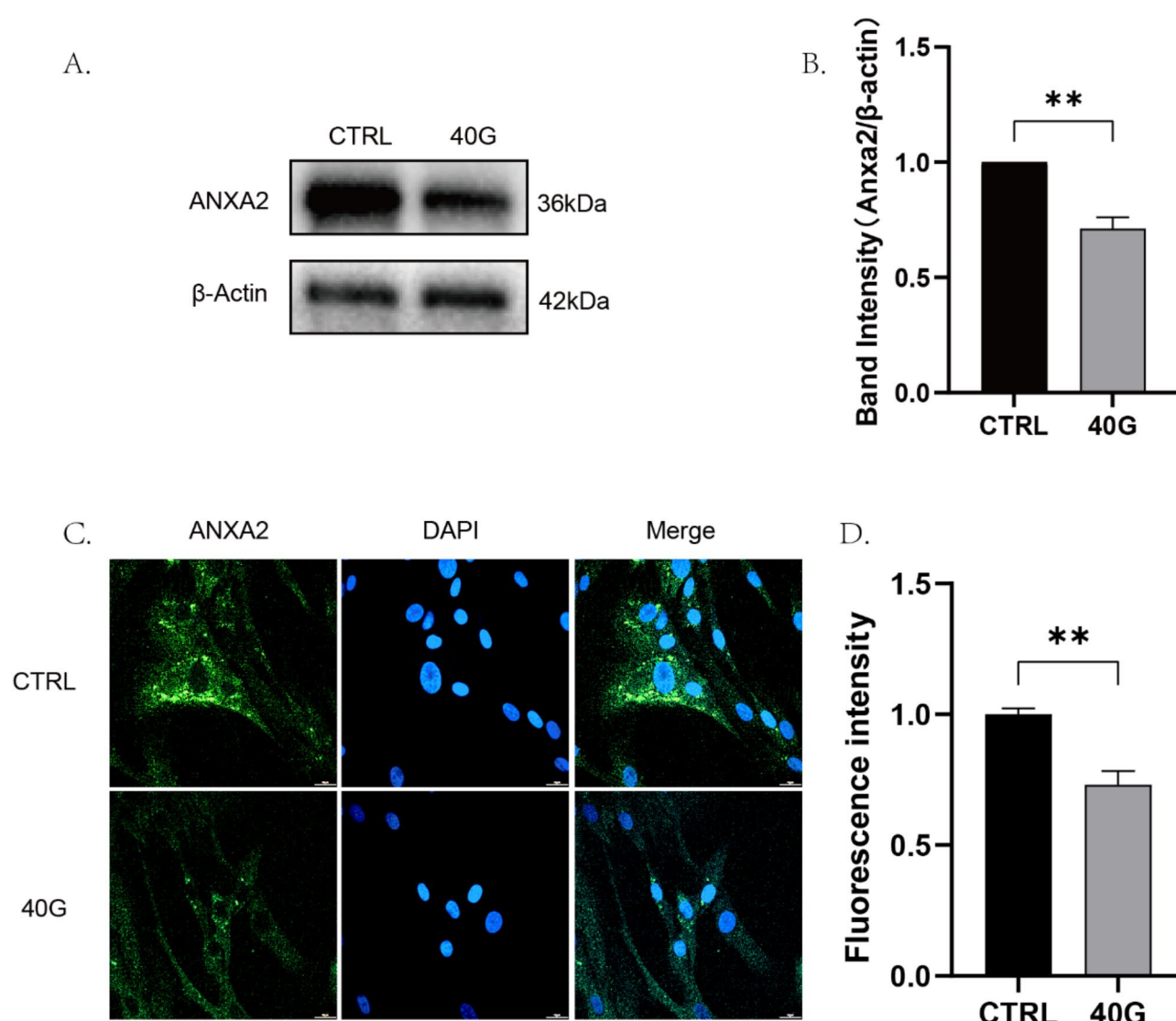


Fig. 1. The expression of ANXA2 in PDLCS under high glucose conditions. **(A)** The expression of ANXA2 at protein level in PDLCS. **(B)** Semi-quantitative analysis of ANXA2. **(C)** Representative confocal images of ANXA2 staining. **(D)** The relative fluorescence intensity of ANXA2 in CTRL and 40G. The intensity of ANXA2 were quantified by using ImageJ. All data are presented as the means $\pm$ SEM from three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. CTRL: Control; 40G: 40mM glucose.

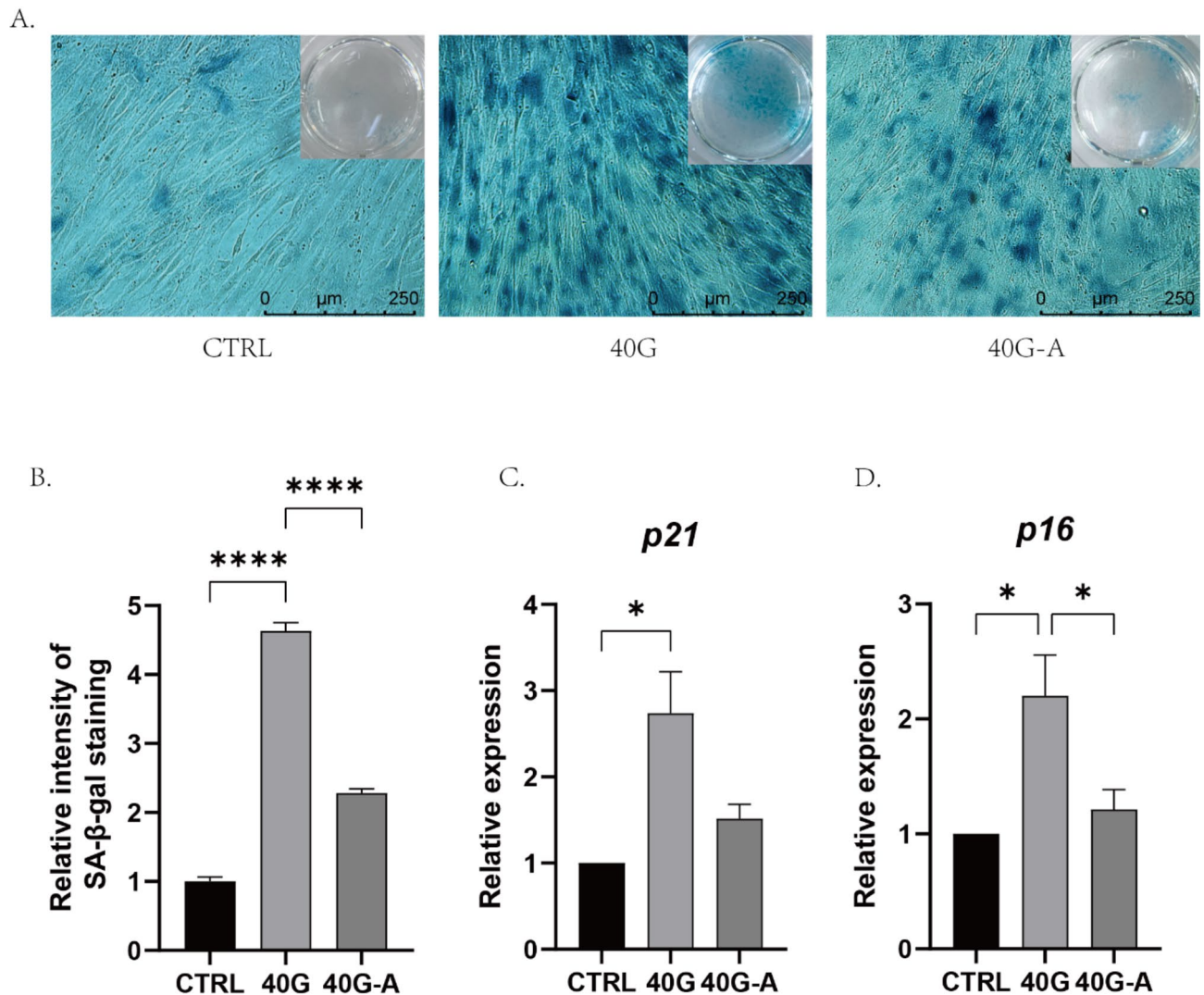


Fig. 2. The effect of high glucose and ANXA2 on cellular senescence of PDLCs. PDLCs were transfected with pcDNA3.1-ANXA2 or its empty vector controls, followed by treatment with high glucose. (A) The SA-β-gal staining of PDLCs. (B) Semi-quantitative analysis of SA-β-gal staining was performed by an Image J Analyzer. (C,D) The mRNAs of *p21*, *p16* were evaluated by qRT-PCR. All data are presented as the means ± SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. CTRL: Control; 40G: 40mM glucose; 40G-A: 40mM glucose + ANXA2 overexpression.

ANXA2 improves mitochondrial function of PDLCs in high glucose condition

ROS

Mitochondria are the main sites of ROS production, and when mitochondria are dysfunctional, ROS are produced in large quantities²⁰. We found a significant increase in ROS in the high-glucose group (40G) compared with the control group (CTRL), and a significant decrease in ROS production in the group overexpressing ANXA2 (40G-A). (Fig. 3A-B)

Mitochondrial morphology

High glucose-induced mitochondrial dysfunction has been implicated in human cellular senescence¹⁷. Therefore, we hypothesized that the inhibition of cellular senescence in PDLCs by ANXA2 in high glucose may be partly due to improved mitochondrial function. Unlike the regularly distributed rod-shaped or elongated mitochondria in the control group, the mitochondria in the high glucose group (40G) were fragmented, aberrant, and blister-like, whereas the above conditions were significantly reduced in the group overexpressing ANXA2 (40G-A). (Fig. 3C)

Mitophagy

At the same time, we wanted to know whether ANXA2 is associated with mitophagy. Then we detected mitophagy-related proteins. Compared to 40G, 40G-A had a significantly higher LC3-II/I ratio and Parkin expression, and a significant decrease in p62, an established autophagy substrate (Fig. 4A-E). Increased levels of LC 3B-II indicate either an increase in autophagosome formation or a decrease in autophagosome degradation^{21,22}. To check for

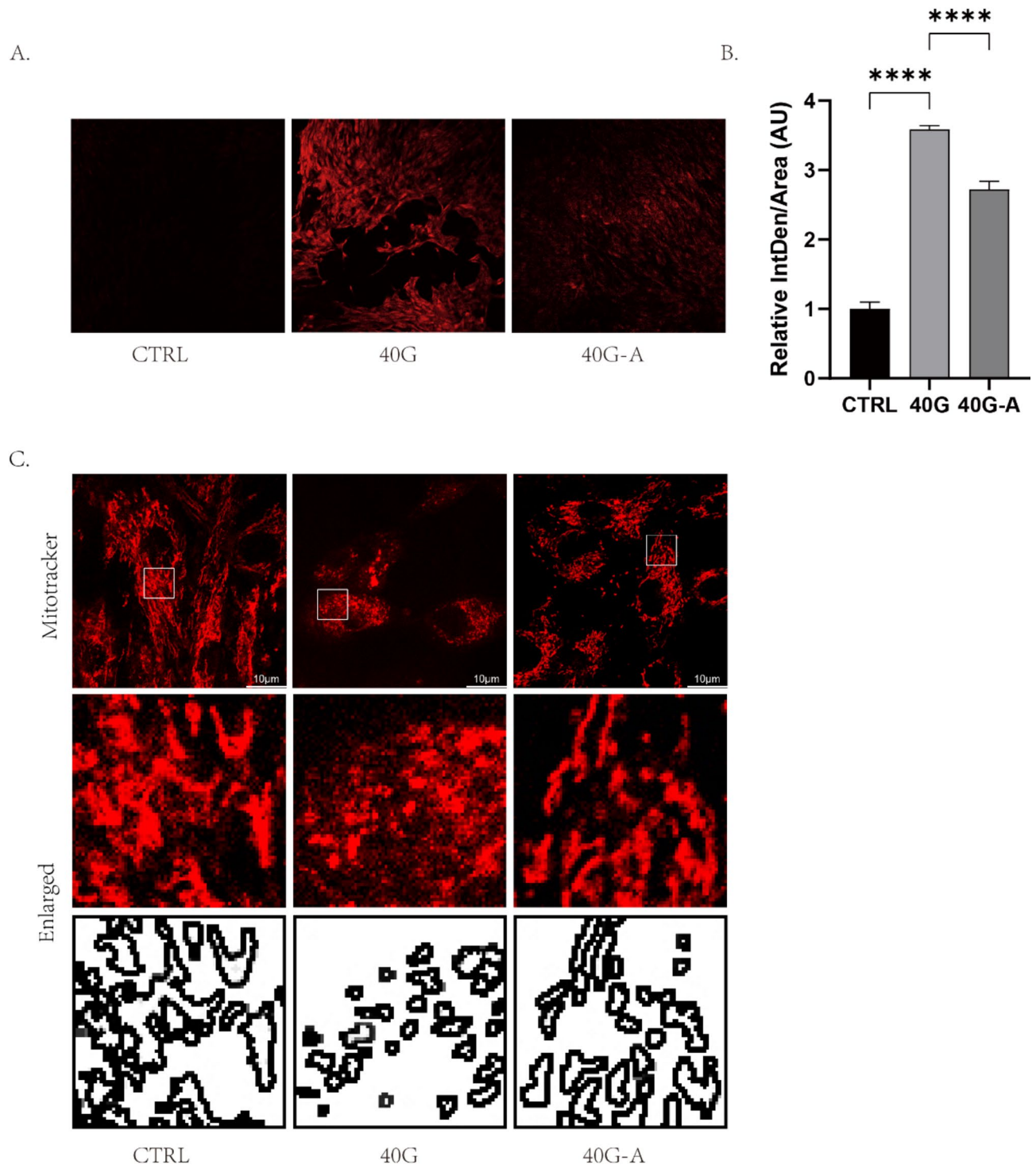


Fig. 3. The effect of high glucose and ANXA2 on ROS production and mitochondrial morphology of PDLCs. PDLCs were transfected with pcDNA3.1-ANXA2 or its empty vector controls, followed by treatment with high glucose. (A) The ROS accumulation of PDLCs. (B) Semi-quantitative analysis of ROS accumulation. (C) Representative images of mitochondrial morphology. All data are presented as the means $\pm$ SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. CTRL: Control; 40G: 40mM glucose; 40G-A: 40mM glucose + ANXA2 overexpression.

both possibilities, PDLCs were treated with chloroquine, an autophagosome-lysosome fusion inhibitor, which inhibits autophagosome degradation, leading to the accumulation of LC 3B-II. We found that LC3-II/I was significantly increased in the CQ-added group compared with the unadded group (Fig. 4E-F), suggesting that

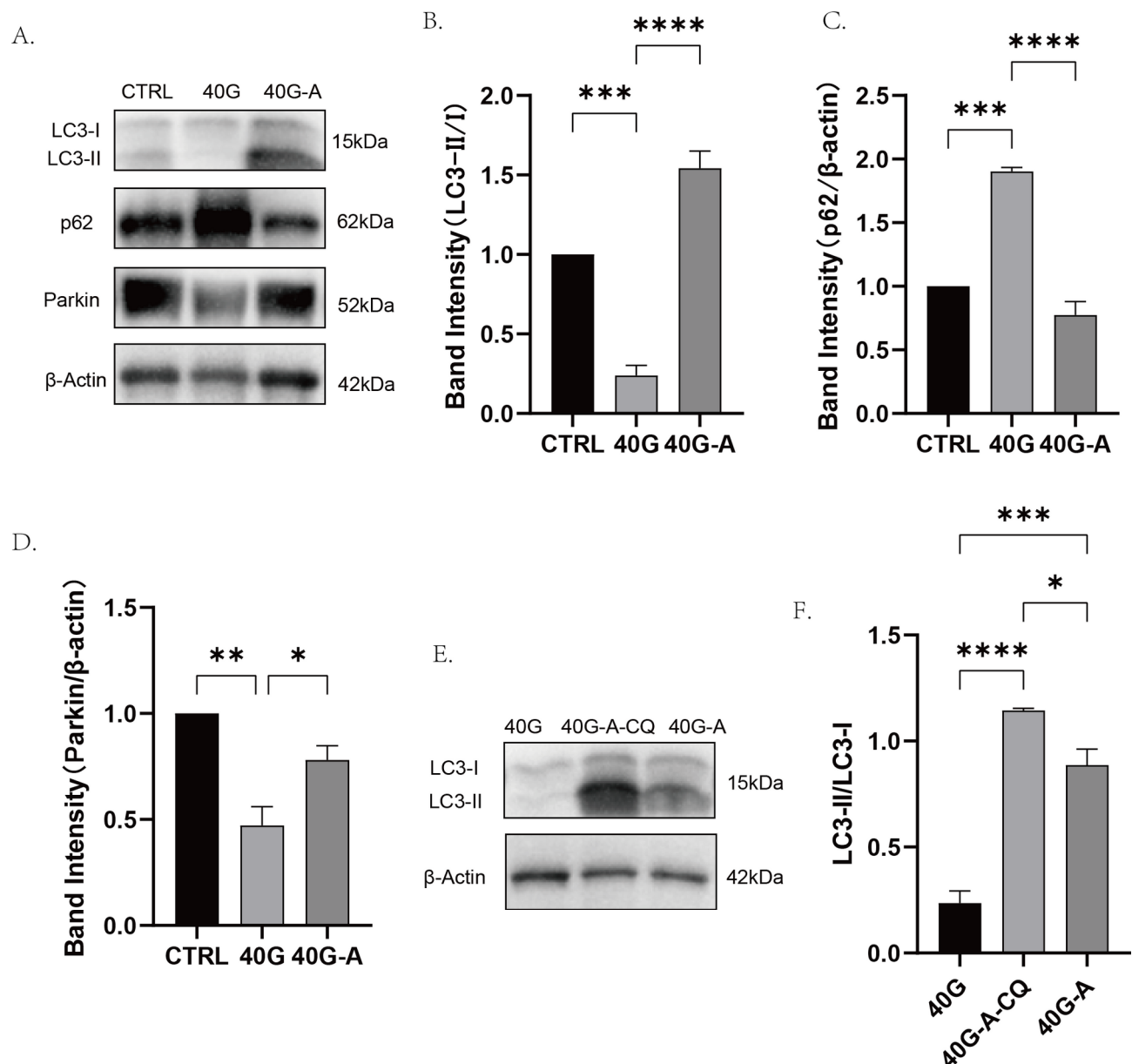


Fig. 4. The effect of high glucose and ANXA2 on Mitophagy of PDLs. PDLs were transfected with pcDNA3.1-ANXA2 or its empty vector controls, followed by treatment with high glucose or CQ (chloroquine, 10 μ M) (A) The expression of LC3, p62, Parkin at protein level in PDLs. (B–D) Semi-quantitative analysis of LC3-II/I, p62, Parkin. (E) The expression of LC3 protein level in PDLs with CQ. (F) Semi-quantitative analysis of LC3-II/I. All data are presented as the means $\pm$ SEM from three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001. CTRL: Control; 40G: 40mM glucose; 40G-A: 40mM glucose + ANXA2 overexpression. 40G-A-CQ: 40mM glucose + ANXA2 overexpression + chloroquine.

the increase in the LC3-II/I ratio after overexpression of ANXA2 was not caused by a decrease in autophagosome degradation.

In summary, ANXA2 can improve the mitochondrial function of PDLs in a high glucose environment.

ANXA2 regulates the AKT/eNOS signaling pathway

Since AKT/eNOS is widely involved in the regulation of mitochondrial function²³, we hypothesized that ANXA2 regulates mitochondrial function through the AKT/eNOS signaling pathway. Western blotting assays showed that p-AKT/AKT and p-eNOS/eNOS were significantly reduced after siRNA knockdown of ANXA2 (Fig. 5A–D). In contrast, p-AKT/AKT and p-eNOS/eNOS were significantly increased after overexpression of ANXA2 (Fig. 5E–H). It was demonstrated by both forward and reverse that ANXA2 could regulate the AKT/eNOS pathway.

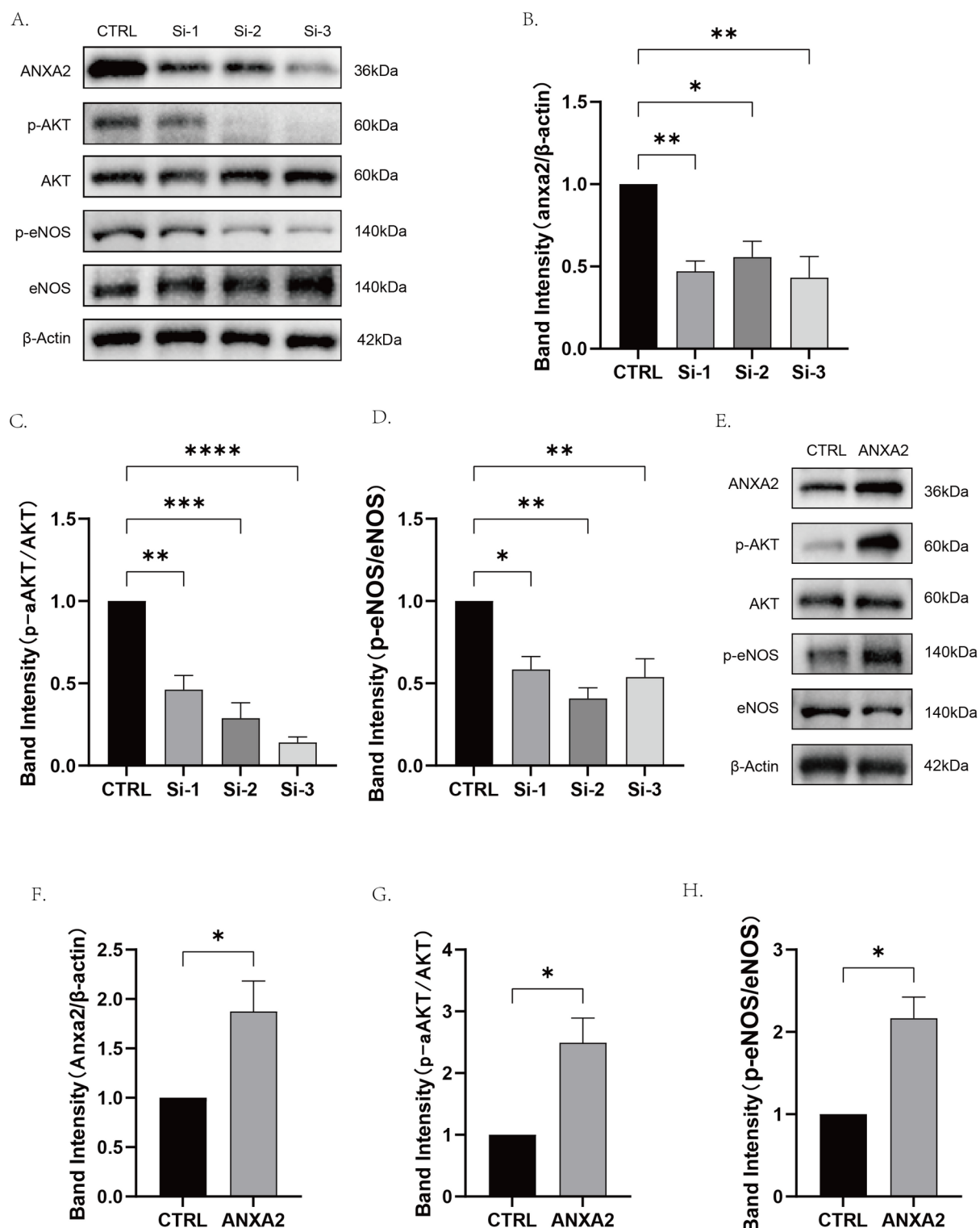


Fig. 5. The effect of ANXA2 on AKT/eNOS signaling pathway. PDLCs were transfected with siRNA, pcDNA3.1-ANXA2 or its empty vector controls. (A) The expression of Akt, p-Akt (Ser473), eNOS, p-eNOS(Ser1177) at protein level in PDLCs after knockdown of ANXA2. (B–D) Semi-quantitative analysis of ANXA2, p-Akt/Akt, p-eNOS/ eNOS. (E) The expression of Akt, p-Akt (Ser473), eNOS, p-eNOS(Ser1177) at protein level in PDLCs after overexpression of ANXA2. (F–H) Semi-quantitative analysis of ANXA2, p-Akt/Akt, p-eNOS/ eNOS. All data are presented as the means $\pm$ SEM from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

ANXA2 inhibits cellular senescence of PDLs in high glucose condition through the AKT/eNOS signaling pathway

To verify whether the inhibition of cellular senescence of PDLs by ANXA2 is through the AKT/eNOS signaling pathway, we used the MK2206(AKT inhibitor) and L-NAME (eNOS inhibitor). As we can see, compared to 40-A, the group with these two inhibitors had higher β -galactosidase activity(Fig. 6A-B)and higher expression of

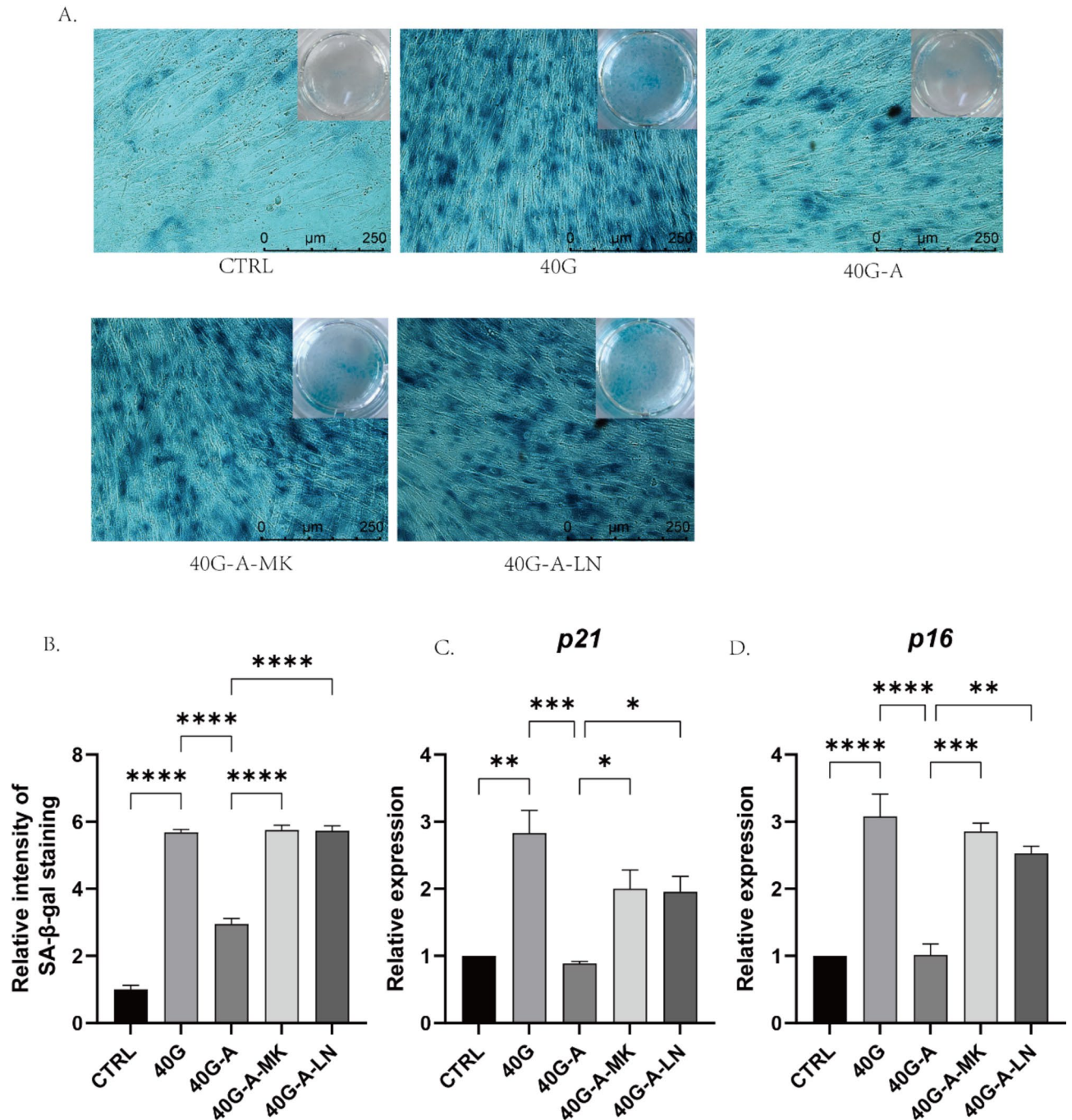


Fig. 6. The effect of ANXA2/AKT/eNOS signaling pathway on cellular senescence of PDLs under high glucose conditions. PDLs were transfected with pcDNA3.1-ANXA2 or its empty vector controls, followed by treatment with high glucose, MK2206(10 μ M)(AKT inhibitor) and L-NAME (100 μ M)(eNOS inhibitor) (A) The SA- β -gal staining of PDLs. (B) Semi-quantitative analysis of SA- β -gal staining was performed by an Image J Analyzer. (C,D) The mRNAs of *p21*, *p16* were evaluated by qRT-PCR. All data are presented as the means $\pm$ SEM from three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001. CTRL: Control; 40G: 40mM glucose; 40G-A: 40mM glucose + ANXA2 overexpression; 40G-A-MK: 40mM glucose + ANXA2 overexpression + MK2206(AKT inhibitor); 40G-A-LN: 40mM glucose + ANXA2 overexpression + L-NAME (eNOS inhibitor).

senescence-related genes *p21, p16* (Fig. 6C–D). It can be concluded that ANXA2 inhibits cellular senescence of PDLs in high glucose environment through AKT/eNOS signaling pathway.

ANXA2 regulates mitochondrial function in PDLs in a high glucose environment through the AKT/eNOS signaling pathway

To test whether ANXA2 regulates mitochondrial function through the AKT/eNOS signaling pathway, we used the MK2206 (AKT inhibitor) and L-NAME (eNOS inhibitor). We found that compared to 40G-A, the group to which both of these inhibitors were added had more ROS production (Fig. 7A–B), abnormal mitochondrial morphology (Fig. 7C), and inhibited mitophagy (Fig. 8A–D). Thus, ANXA2 can regulate mitochondrial function through the AKT/eNOS signaling pathway.

Discussion

Diabetes mellitus is one of the risk factors for periodontitis, yet the underlying mechanisms of action are not yet clear. In this study, we emphasized cellular senescence and mitochondrial dysfunction as potential links between diabetes and periodontitis. First, we observed that ANXA2 expression was down-regulated in PDLs under high glucose conditions. Furthermore, overexpression of ANXA2 delayed high glucose-induced cellular senescence and mitochondrial dysfunction. Finally, we confirmed that ANXA2 mediated the AKT/eNOS signaling pathway to regulate high glucose-induced mitochondrial dysfunction and cellular senescence in PDLs.

Inflammation bridges the gap between diabetes and periodontitis. Senescent cells may be one of the sources of chronic systemic inflammation: the senescence-associated secretory phenotype (SASP) is a group of proinflammatory cytokines, chemokines, and proteases secreted by senescent cells^{9,24,25}, which collectively modify the local environment. When cells senesce, cellular function is severely impaired (impairing tissue function in an autocrine manner)²⁶; in addition, senescent cells can impair the function of neighboring cells by senescing them²⁷ or by destroying their differentiation potential^{28,29} (impairing tissue function in a paracrine manner); regardless of how cellular senescence is induced, senescent cells actively secrete SASP components.

Periodontal ligament cells (PDLs), a major component of the periodontal membrane including fibroblasts, osteoblasts, osteoclasts, macrophages, undifferentiated mesenchymal cells, and many other components, are the main target of inflammatory attack in periodontitis and are closely related to periodontal tissue health³⁰. It has been demonstrated that senescent PDLs secrete SASP components, including inflammatory cytokines, chemokines, and MMPs/TIMPs, leading to inflammation and destruction of periodontal tissues³¹. In addition, cellular senescence causes a decrease in immune function, wound healing ability, and bone tissue regeneration potential of periodontal tissues³². And we found that ANXA2 can regulate high glucose-induced cellular senescence in PDLs, which can be hypothesized to have great research potential in the association of periodontitis and diabetes.

Mitochondrial dysfunction is considered a marker and inducer of cellular senescence^{33,34}. Mitochondria provide essential energy and substrates for cellular metabolism and growth through the respiratory process^{35–38}. However, aberrant mitochondria lead to reduced energy supply and a massive increase in ROS release, which leads to disruption of mitochondrial endohomeostasis and cellular senescence^{33,39,40}. It has been reported that sustained high glucose stimulation leads to impaired mitochondrial electron transport and promotes high ROS production, which in turn causes damage to the mitochondria themselves and mitochondrial DNA (mtDNA), leading to apoptosis and activation of downstream inflammatory signaling pathways^{41–43}. Interestingly, in the present experiments, we found that high glucose-induced mitochondrial dysfunction was associated with ANXA2 depletion. Coincidentally, it has recently been shown that TIM-4 promotes mitochondrial function and homeostasis through activation of the PI3K/AKT signaling pathway by binding to ANXA2 and thus promotes cell proliferation⁴⁴. In addition, the interaction of ANXA2 with FAXC to promote mitochondrial homeostasis and thus the progression of cholangiocarcinoma has been reported⁴⁵. This supports our results to a certain extent. Current attention to ANXA2 has focused on the progression of various cancers^{46–48}. As an intracellular calcium-dependent phospholipid-binding protein involved in a variety of biological functions^{49–51}, its role in various metabolic disorders and chronic inflammation is also worthy of being explored^{16,52–54}.

Candidate genes downstream of PI3K/AKT/eNOS signaling directly regulate mitochondria, controlling mitochondrial function, oxidative stress, and apoptosis^{55,56}. The nonsteroidal salicylic acid receptor antagonist Finerenone (FIN) has been reported to ameliorate mitochondrial dysfunction via the PI3K/Akt/eNOS signaling pathway in diabetic tubulopathy²³. Interestingly, we found that ANXA2 can regulate mitochondrial function via the AKT/eNOS axis in a high glucose environment. Current research focuses on in vitro experiments and lacks in vivo experiments. In future studies, we will conduct in vivo experiments. We will build a rat model with both diabetes and periodontitis, then overexpress and silence ANXA2 in some of the rats, and then test the destruction of periodontal tissues to confirm the therapeutic effect of ANXA2.

Conclusion

In conclusion, we found a decreased expression of ANXA2 in high glucose environment. And the depletion of ANXA2 may be one of the causes of mitochondrial dysfunction and cellular senescence in PDLs induced by high glucose. Importantly, we report a previously unreported mechanism in which ANXA2 maintains mitochondrial homeostasis and delays cellular senescence in PDLs through the AKT/eNOS signaling pathway. These findings provide new insights into the mechanisms of action in diabetes and periodontitis.

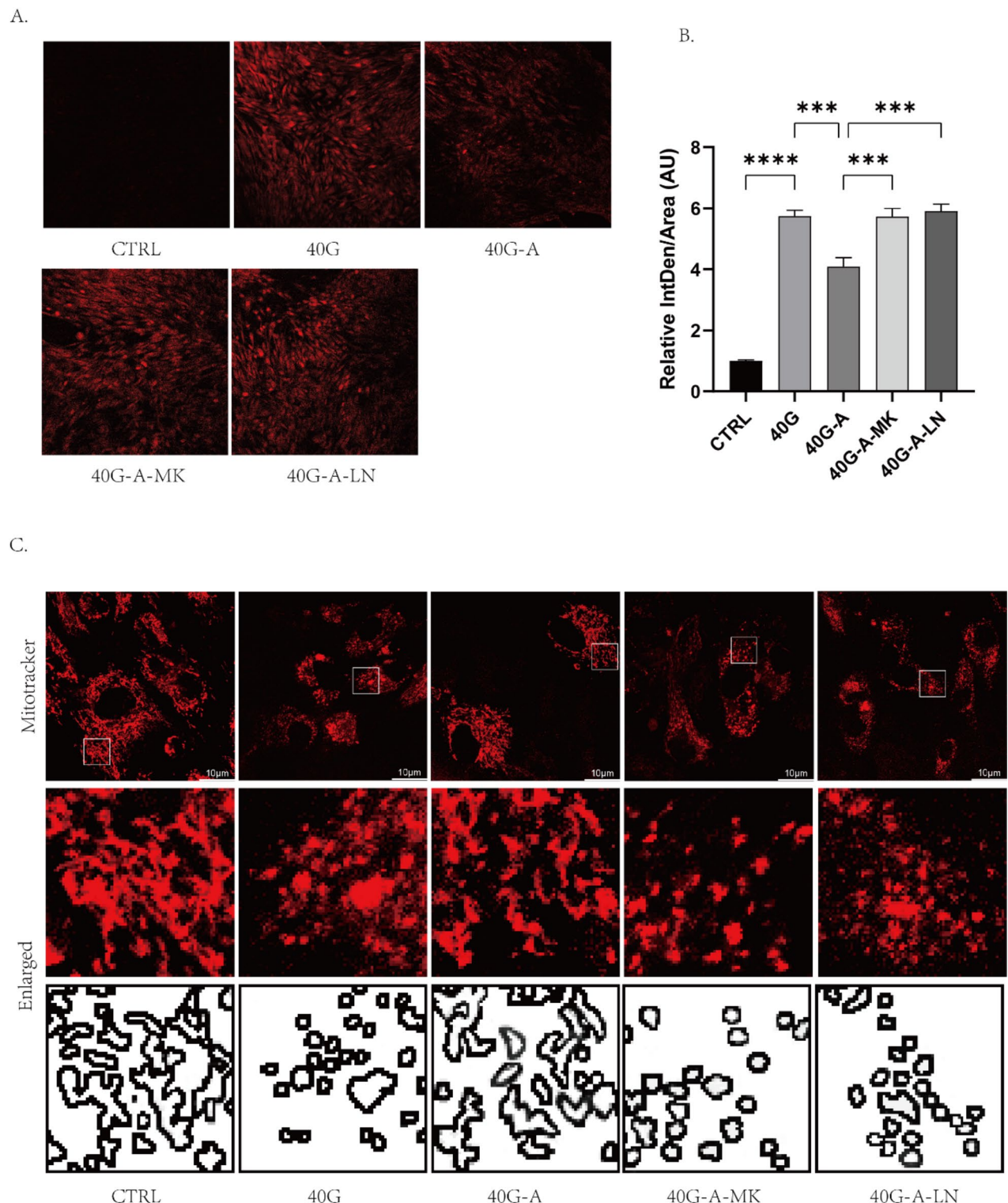


Fig. 7. The effect of ANXA2/AKT/eNOS signaling pathway on ROS production and mitochondrial morphology of PDLCs under high glucose conditions. PDLCs were transfected with pcDNA3.1-ANXA2 or its empty vector controls, followed by treatment with high glucose, MK2206(10 μ M)(AKT inhibitor) and L-NAME (100 μ M)(eNOS inhibitor). **(A)** The ROS accumulation of PDLCs. **(B)** Semi-quantitative analysis of ROS accumulation. **(C)** Representative images of mitochondrial morphology. All data are presented as the means $\pm$ SEM from three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001. CTRL: Control; 40G: 40mM glucose; 40G-A: 40mM glucose + ANXA2 overexpression. CTRL: Control; 40G: 40mM glucose; 40G-A: 40mM glucose + ANXA2 overexpression; 40G-A-MK: 40mM glucose + ANXA2 overexpression + MK2206(AKT inhibitor); 40G-A-LN: 40mM glucose + ANXA2 overexpression + L-NAME (eNOS inhibitor).

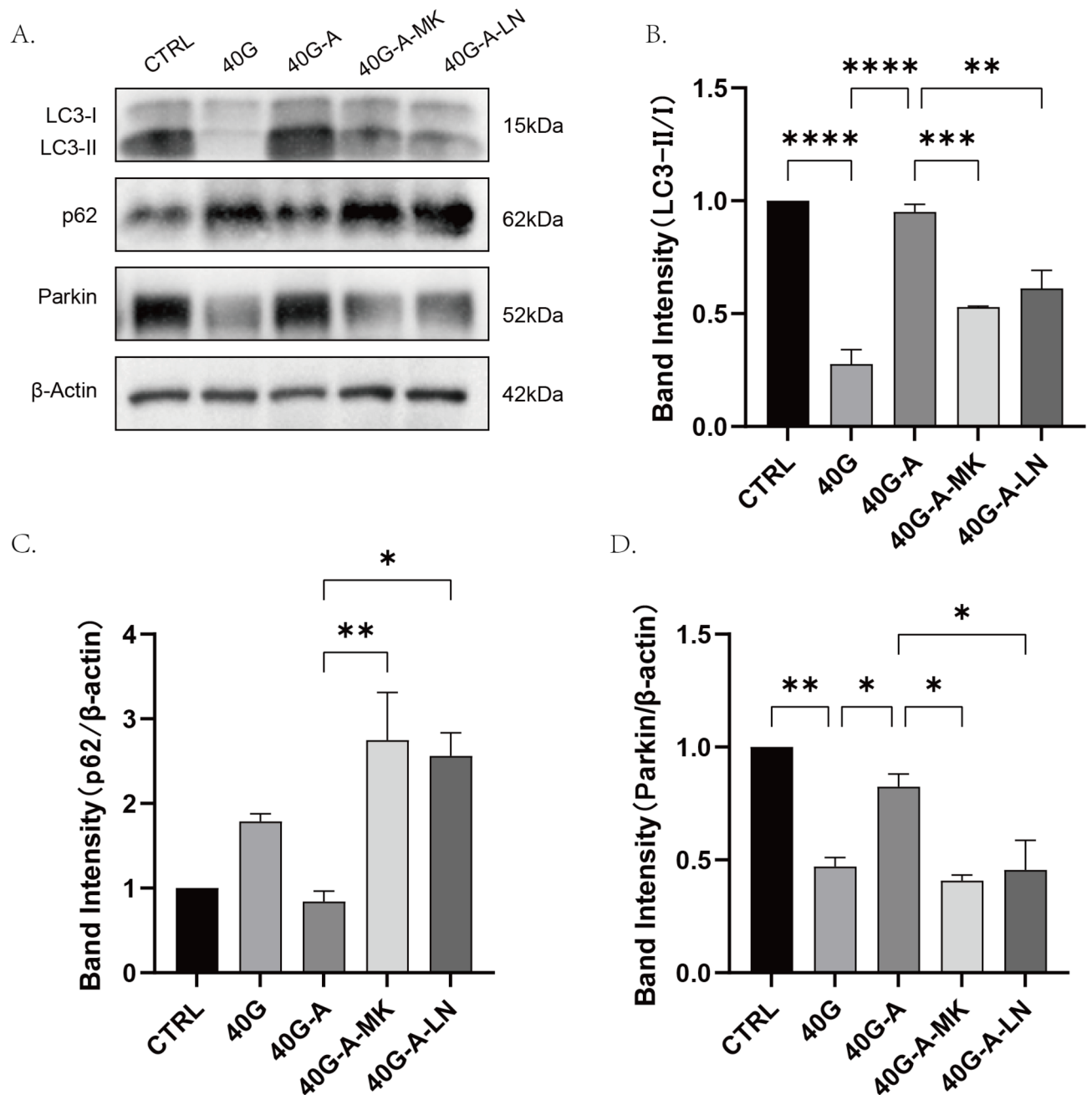


Fig. 8. The effect of ANXA2/AKT/eNOS signaling pathway on Mitophagy of PDLs under high glucose conditions. PDLs were transfected with pcDNA3.1-ANXA2 or its empty vector controls, followed by treatment with high glucose, MK2206(10 μ M)(AKT inhibitor) and L-NAME (100 μ M)(eNOS inhibitor). **(A)** The expression of LC3, p62, Parkin at protein level in PDLs. **(B–D)** Semi-quantitative analysis of LC3-II/I, p62, Parkin. All data are presented as the means $\pm$ SEM from three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001. CTRL: Control; 40G: 40mM glucose; 40G-A: 40mM glucose + ANXA2 overexpression. CTRL: Control; 40G: 40mM glucose; 40G-A: 40mM glucose + ANXA2 overexpression; 40G-A-MK: 40mM glucose + ANXA2 overexpression + MK2206(AKT inhibitor); 40G-A-LN: 40mM glucose + ANXA2 overexpression + L-NAME (eNOS inhibitor).

Data availability

The authors declare that majority of the data supporting the findings of this study are available within the paper and its supplementary information files. More detailed specific data is available from the corresponding author on request.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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