ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
In press.

Inhibition of LTBP2 Suppresses High Glucose-Induced Proliferation, Fibrosis, and Inflammation in Glomerular Mesangial Cells by Disrupting the PI3K/Akt/NF-κB Pathway

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Received: 19 October 2024; Received in revised form: 28 November 2024; Accepted: 9 December 2024

ABSTRACT

Latent transforming growth factor-β binding protein-2 (LTBP2) plays a significant role in tissue fibrosis. This research aimed to elucidate whether LTBP2 influences the progression of diabetic nephropathy (DN) through the phosphatidylinositol 3-kinases/protein kinase B (PI3K/Akt)/nuclear factor kappa-B (NF-κB) pathway.

The HBZY-1 cells were exposed to high glucose to create diabetic nephropathy cell model. LTBP2 levels were examined by Western blot and immunofluorescence. After verifying the transfection efficiency of si-LTBP2, cell counting kit-8, 5-ethynyl-2-deoxyuridine staining, Western blot, flow cytometry and immunofluorescence were utilized to assess the proliferation, apoptosis and fibrosis of HBZY-1 cells, respectively. Collagen deposition was also detected by Sirius red staining, and inflammatory factors levels were determined by Elisa. PI3K/Akt/NF-κB pathway activators were applied to explore whether LTBP2 silencing could play a role in DN by modulating this pathway.

After treatment with high glucose, the expression of LTBP2 was elevated in HBZY-1 cells. LTBP2 silencing hindered the aberrant proliferation of HBZY-1 cells, with no significant effect on apoptosis; meanwhile, it reduced fibrosis, decreased collagen content, and decreased inflammatory factors levels in HBZY-1 cells. Following treatment with high glucose, the PI3K, Akt, and p65 phosphorylation levels were increased, whereas silencing LTBP2 reduced them. Activators of the PI3K/Akt/NF-κB pathway weakened the inhibition of LTBP2 silencing on cell proliferation, fibrosis, and inflammation.

In conclusion, silencing of LTBP2 weakened the proliferation, fibrosis, and inflammation of HBZY-1 cells treated with high glucose by hindering the PI3K/Akt/NF-κB pathway. This research offers a new reference for the targeted therapy of DN.

Keywords: Diabetic nephropathy; Inflammation; Latent transforming growth factor-β binding protein-2; Mesangial cell; Phosphatidylinositol 3-kinases/protein kinase B/nuclear factor kappa-B pathway

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INTRODUCTION

Diabetes is a chronic metabolic disorder marked by continuously high levels of blood glucose.[1] According to

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statistics, in 2021, 536.6 million adults worldwide were affected by diabetes, and by 2045, the number of adult diabetics will rise to 783.2 million.[2] About 40% of individuals with type 2 diabetes progressively develop diabetic nephropathy (DN) worldwide, with its incidence increasing every year.[3] DN involves a complex pathologic process in which high glucose is central to its onset and progression, affecting all types of cells, including mesangial cells, podocytes, and inflammatory cells.[4,5] Early symptoms of DN are not obvious and are often diagnosed when they enter a large amount of proteinuria is present. This condition is difficult to reverse and can even develop into end-stage renal disease, which can only be treated with kidney transplantation.[6,7] Therefore, the search for specific biomarkers is crucial for early diagnosis and targeted therapy in patients with DN.

When exposed to elevated glucose levels, glomerular mesangial cells generate significant levels of reactive oxygen species (ROS), prompting the release of inflammatory mediators and cytokines by the immune system. This leads to excessive cell proliferation and hypertrophy, along with the overproduction of extracellular matrix (ECM), resulting in interstitial fibrosis.[8,9] Therefore, inflammation, cellular hyperproliferation, and fibrosis can be considered important pathological mechanisms in DN. Latent TGF-β binding protein-2 (LTBP2), encoded by the protofibrillar protein LTBP gene family, plays a significant role in the ECM.[10,11] It has been demonstrated that LTBP2 is implicated in the fibrotic process in various tissues, including cardiac fibrosis,[12] pulmonary fibrosis,[13] and oral submucosal fibrosis.[14] Down-regulation of LTBP2 expression has been shown to ameliorate renal aging, which can induce renal fibrosis, suggesting that LTBP2 may be associated with renal fibrosis.[15] However, so far, there is no clear information about the effect of LTBP2 on DN.

The phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway serves as a key link between external signals and cellular responses and is crucial in regulating cell proliferation, apoptosis, and oxidative stress.[16,17] PI3K is normally activated following growth factor interaction with receptor tyrosine kinases, Akt is a downstream target of PI3K and is recruited to the plasma membrane and phosphorylated following PI3K activation.[18] Nuclear factor kappa-B (NF-κB) is essential in regulating both inflammatory and immune responses, with its expression and activity being modulated by the PI3K/Akt pathway.[19,20] Notably, LTBP2 is involved in regulating the PI3K/Akt and NF-κB pathways.[21,22] Therefore, we hypothesized that LTBP2 may impact DN progression by modulating the PI3K/Akt/NF-κB pathway. Based on this hypothesis, the present research constructs a DN cell model and investigates whether LTBP2 influences cell proliferation, fibrosis, and inflammation triggered by high glucose. This research aims to elucidate the impact of LTBP2 on DN and its action mechanism, to provide a new reference for targeted therapy of DN.

MATERIALS AND METHODS

Cell Culture and Processing

Rat glomerular mesangial cell line HBZY-1 was purchased from Pricella Biotechnology Co., Ltd. (Wuhan, Hubei, China). DMEM medium (Gibco, Grand Island, NY, USA) was enriched with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin double antibody (Gibco, Grand Island, NY, USA) and served as the cell culture medium. The cell culture was maintaines at 37°C with 5% CO_2. Referring to the method of Dong et al[23] a culture medium containing 5.6 mM glucose (G8150, Solarbio, Beijing, China) was used to culture HBZY-1 cells, which was recorded as normal glucose (NG) group. To control the osmotic pressure, a culture medium added 5.6 mM glucose + 24.4 mM mannitol was used to culture HBZY-1 cells, which was recorded as the mannitol (MA) group. After 48 hours of incubation, subsequent tests were performed. A culture medium with 30 mM glucose was used to culture HBZY-1 cells for 12, 24, or 48 h, which was recorded as high glucose (HG) group. In addition, the PI3K/Akt activator 740 Y-P (30 μM, HY-P0175, MedChemExpress, Monmouth Junction, NJ, USA) or the NF-κB activator PMA (1 μg/mL, HY-18739, MedChemExpress, Monmouth Junction, NJ, USA) was added 30 min before modeling in the HG group.

Cell Transfection

Green fluorescent protein (GFP)-tagged LTBP2 silencing plasmid (si-LTBP2) and negative control (si-NC) were synthesized by RiboBio Co., Ltd. (Guangzhou, Guangdong, China). HBZY-1 cells were inoculated into 24-well plates (1×10^4 cells/well), and the plasmids mentioned above were transfected into HK-2 cells when the cell confluence reached 50%. The transfection procedure for these plasmids was carried

out following the Lipofectamine 3000 instructions (Invitrogen, Carlsbad, CA, USA). Cell transfection efficiency was assessed by observing the green fluorescence of the cells after 48 hours of transfection utilizing a fluorescence-inverted microscope (MF52-N, Guangzhou Ming-Mei Technology Co., Ltd, Guangdong, China).

RT-qPCR

The total RNA of HBZY-1 cells was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and subsequently, reverse transcription was performed by adding AMV reverse transcriptase (TAKARA, Tokyo, Japan) to obtain cDNA. Following that, PCR amplification was conducted using TB Green® Premix Ex Taq™ II (TAKARA, Tokyo, Japan)[24]. The internal reference used in the analysis was GAPDH[25].

The following are the primer sequences used in this experiment: *LTBP2*: F: 5'-GCTACACTTGTGACTGCTT-3'; R: 5'-GCGATAGGAACCCTCTGT-3'. *GAPDH*: F: 5'-GCAAGTTCAACGGCACAG-3'; R: 5'-GCCAGTAGACTCCACGACA-3'.

Immunofluorescence

HBZY-1 cells after different treatments were placed in culture dishes at 2×10^4/mL. Once the cells reached a density of 50% to 60%, the culture fluid in the dishes was aspirated, and the cells were exposed to 4% paraformaldehyde (158127, Sigma-Aldrich, St. Louis, MO, USA) for 20 min. The cells were covered with 0.3% Tritonx-100 (T8787, Sigma-Aldrich, St. Louis, MO, USA) for 10 min, then blocked by adding bovine serum albumin (B2064, Sigma-Aldrich, St. Louis, MO, USA) for 40 min, and incubated at 4°C for overnight with LTBP2 primary antibody (PA5-51930, 1:100, Invitrogen, Carlsbad, CA, USA), fibronectin (FN) primary antibody (ab2413, 1:100, Abcam, Cambridge, MA, USA), collagen I (Col I) primary antibody (ab34710, 1:200, Abcam, Cambridge, MA, USA) or collagen IV (Col IV) primary antibody (ab6586, 1:100, Abcam, Cambridge, MA, USA). On the following day, after adding FITC-labeled goat anti-rabbit IgG (31460, 1:10000, Invitrogen, Carlsbad, CA, USA), the mixture was incubated in darkness for 1 h. Finally, exposed to DAPI solution (Beyotime, Shanghai, China) and left to incubate away from light for 10 min, and the development was observed through fluorescence microscopy. Fluorescence intensity was obtained after processing the images with Image J software (Wavne Resband, National Institute of Mental Health, USA).

Cell Counting Kit-8 (CCK-8) Assay

HBZY-1 cells were seeded in a 96-well cell culture plate (1.5×10^4cells/well), and after the cell adhesion, the original medium was discarded and exchanged for 200 μL of medium with 10% CCK-8 reagent (C0038, Beyotime, Shanghai, China). Following 2-hour incubation at 37°C in a light-protected incubator, the OD_{450} values of the cells were assessed by a microplate reader (1410101, Thermo Fisher Scientific, Waltham, MA, USA), and the cells' survival was observed under a light microscope (CKX53, Olympus, Tokyo, Japan).

5-Ethynyl-2-deoxyuridine (EdU) Staining

Detection of HBZY-1 cell proliferation was carried out utilizing the EdU cell proliferation detection kit (C0071S, Beyotime, Shanghai, China). Cells with different treatments were cultured for 48 h. After that, 10 μM EdU culture solution and incubated for 1 h away from light, followed by rinsing twice with PBS. Subsequently, they were exposed to 4% paraformaldehyde for 20 min. Added 0.3% Triton X-100 to permeabilize for 10 min, and Click reaction solution (Invitrogen, Carlsbad, CA, USA) was incubated for 30 min away from light. Finally, the cells were exposed to DAPI solution and incubated for 10 min, and the film was blocked by anti-burst, then observed and photographed with a fluorescence microscope (DM3000, Leica, Heidelberg, Germany).

Flow Cytometry

The HBZY-1 cells from various treatment groups were rinsed twice with PBS and gently mixed by adding 500 μL of Binding Buffer. Following that, 5 μL of Annexin-V-FITC (HY-K1073, MedChemExpress) and 5 μL of propidium iodide (ST1569, Beyotime, Shanghai, China) were added and left to incubate for 15 min in the dark. Flow-specific supersampling tubes were used to transfer the samples, and apoptosis was then identified through flow cytometry (BD FACSCalibur™, BD Biosciences, San Jose, CA, USA).

Sirius Red Staining

The HBZY-1 cell slides were exposed to 4% paraformaldehyde for 30 min, then stained with 0.1% Sirius Red (MM1004, Maokangbio, Shanghai, China) for 1 h and rinsed with tap water. Hematoxylin (C0107,

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Beyotime, Shanghai, China) was applied for 1 min and washed off with tap water. After drying naturally, the slides were observed under a microscope. The collagen content of HBZY-1 cells in the images was analyzed using Image J software.

ELISA

The levels of tumor necrosis factor (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin-1β (IL-1β), and IL-6 in HBZY-1 cell supernatants from different treatment groups were quantified by ELISA. The TNF-α (PT516), MCP-1 (PC128), IL-1β (PI303), and IL-6 (PI328) kits were sourced from Beyotime (Shanghai, China). The cell culture supernatant was added to the ELISA well plate and incubated for 2 h. Following two washes with PBS, the corresponding antibody was added and incubated for 1 h. Subsequently, HRP-labeled Streptavidin was added and incubated for 20 min in the dark, followed by incubation with TMB solution for 30 min away from light. After adding and thoroughly mixing the termination solution, the OD_{450} value was measured and used to calculate the concentration.

Western Blot

The total proteins from HBZY-1 cells were extracted with RIPA lysate (P0013B, Beyotime, Shanghai, China), and protein content was assayed utilizing the BCA Kit (P0012, Beyotime, Shanghai, China). Following electrophoresis, the samples were transferred to PVDF membranes (Invitrogen, Carlsbad, CA, USA) and then blocked with skimmed milk for 2 h. After rinsing twice with PBS, the membranes were incubated at 4°C for overnight with LTBP2 primary antibody (1:1000), Cyclin D1 primary antibody (ab134175, 1:10,000, Abcam, Cambridge, MA, USA), proliferating cell nuclear antigen (PCNA) primary antibody (ab29, 1:100, Abcam, Cambridge, MA, USA), FN primary antibody (1:1000), Col I primary antibody (1:2000), Col IV primary antibody (1:1000), PI3K primary antibody (MA1-74183, 1:1000, Invitrogen, Carlsbad, CA, USA), p-PI3K primary antibody (PA5-104853, 1:1000, Invitrogen, Carlsbad, CA, USA), AKT primary antibody (44-609G, 1:1000, Invitrogen, Carlsbad, CA, USA), p-AKT primary antibody (44-621G, 1:1000, Invitrogen, Carlsbad, CA, USA), p65 primary antibody (51-0500, 1:400, Invitrogen, Carlsbad, CA, USA) or p-p65 primary antibody (ab16502, 1:200, Abcam, Cambridge, MA, USA). The following day, the membranes were incubated in HRP-labeled secondary antibody (1:10,000) washed 3 times and then exposed to a gel imaging system (WD-9413B, Beijing Liuyi Biotechnology Co., Ltd., Beijing, China). After the chemiluminescence reagent reaction the grayscale values of each protein band were obtained following processing the images with Image J software, with GAPDH (MA1-16757, 1:1000, Invitrogen, Carlsbad, CA, USA) serving as the internal reference.

Statistical Analysis

Each experiment was carried out at least three times, with the results reported as the mean value along with the respective standard deviation. SPSS 26.0 software (IBM SPSS Statistics 26) was utilized to process and analyze the data statistically. Student's t-test was utilized to evaluate the differences between the two groups. To assess differences among multiple groups, we conducted a one-way analysis of variance. Prism software (GraphPad 9.0) was utilized for plotting. The symbols *, #, and % indicate significant differences compared to the NG and MA groups, HG group, and HG+si-LTBP2 group, respectively ($p<0.05$).

RESULTS

LTBP2 Exhibits High Expression Levels in HBZY-1 Cells Treated with HG

After treatment with high glucose, Western blot findings indicated a notable increase in LTBP2 level in HBZY-1 cells compared to the NG and MA groups, and the effect was time-dependent (Figure 1A-1B). Additionally, the immunofluorescence analysis demonstrated a marked increase in LTBP2 protein levels in the ECM of HBZY-1 cells, consistent with the results obtained from Western blotting (Figure 1C-1D). Since the effect of high glucose treatment for 48 h was the most significant, we selected HBZY-1 cells that were treated for 48 h for further experiments.

Silencing of LTBP2 Attenuates Proliferation in HBZY-1 Cells

The impact of LTBP2 on HBZY-1 cell proliferation was explored through the transfection of si-LTBP2 and the transfection efficiency was examined. The findings indicated that the percentage of green fluorescence-positive cells was above 80% for both si-LTBP2 and si-NC (Figure 2A), suggesting good transfection efficiency. RT-qPCR assay revealed a significant

decrease in *LTBP2* expression in HBZY-1 cells following si-LTBP2 transfection, which which met the requirements for subsequent experiments (Figure 2B). Furthemore, Western blot findings also revealed a notable decline in LTBP2 level (Figure 2C). After exposure to high glucose, CCK-8 assay findings revealed a notable increase in HBZY-1 cell viability, whereas transfection with si-LTBP2 reduced the effect of high glucose treatment on cell viability (Figure 2D-2E). By EdU staining, after high glucose treatment, high glucose treatment resulted in a significant increase in the number of EdU-positive HBZY-1 cells, whereas silencing LTBP2 attenuated the increased cell proliferation caused by high glucose treatment (Figure 2F-2G). Next, we assessed the levels of proliferation-associated proteins, Cyclin D1 and PCNA through Western blot. Following high glucose treatment, Cyclin D1 and PCNA levels in HBZY-1 cells were markedly increased, while silencing LTBP2 attenuated the effects of high glucose (Figure 2H, 2J-2K). Additionally, flow cytometry results demonstrated that neither high glucose treatment nor silencing of LTBP2 had a significant impact on the apoptosis rate (Figure 2I and 2L). These results indicated that high glucose treatment led to significantly higher viability and proliferation of HBZY-1 cells, whereas silencing LTBP2 attenuated the high glucose-induced cell proliferation.

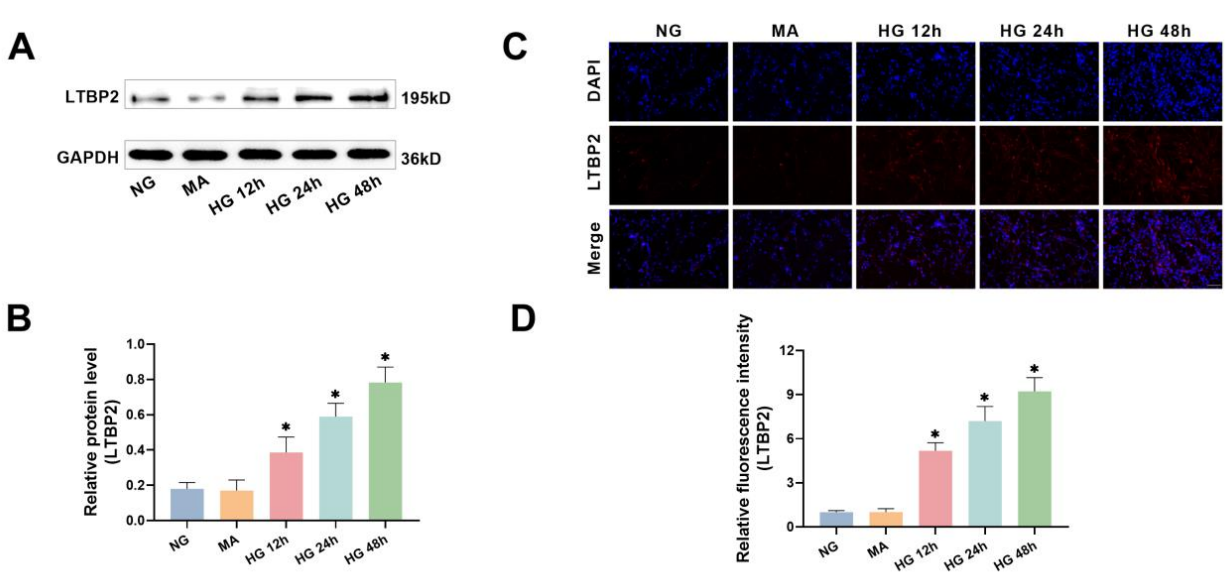

Figure 1. Latent transforming growth factor-β binding protein-2 (LTBP2) exhibits high expression levels in HBZY-1 cells treated with high glucose (HG).

(A-B) Examining LTBP2 level through Western blot. HBZY-1 cells in the normal glucose (NG) group and mannitol (MA) group were incubated with medium added 5.6 mM glucose, 5.6 mM glucose + 24.4 mM mannitol for 48 h, respectively. HBZY-1 cells in the HG group were cultured in culture medium added 30 mM glucose for 12, 24, and 48 h. (C-D) Immunofluorescence for localized expression of LTBP2. n=3. (*p<0.05 vs NG&MA).

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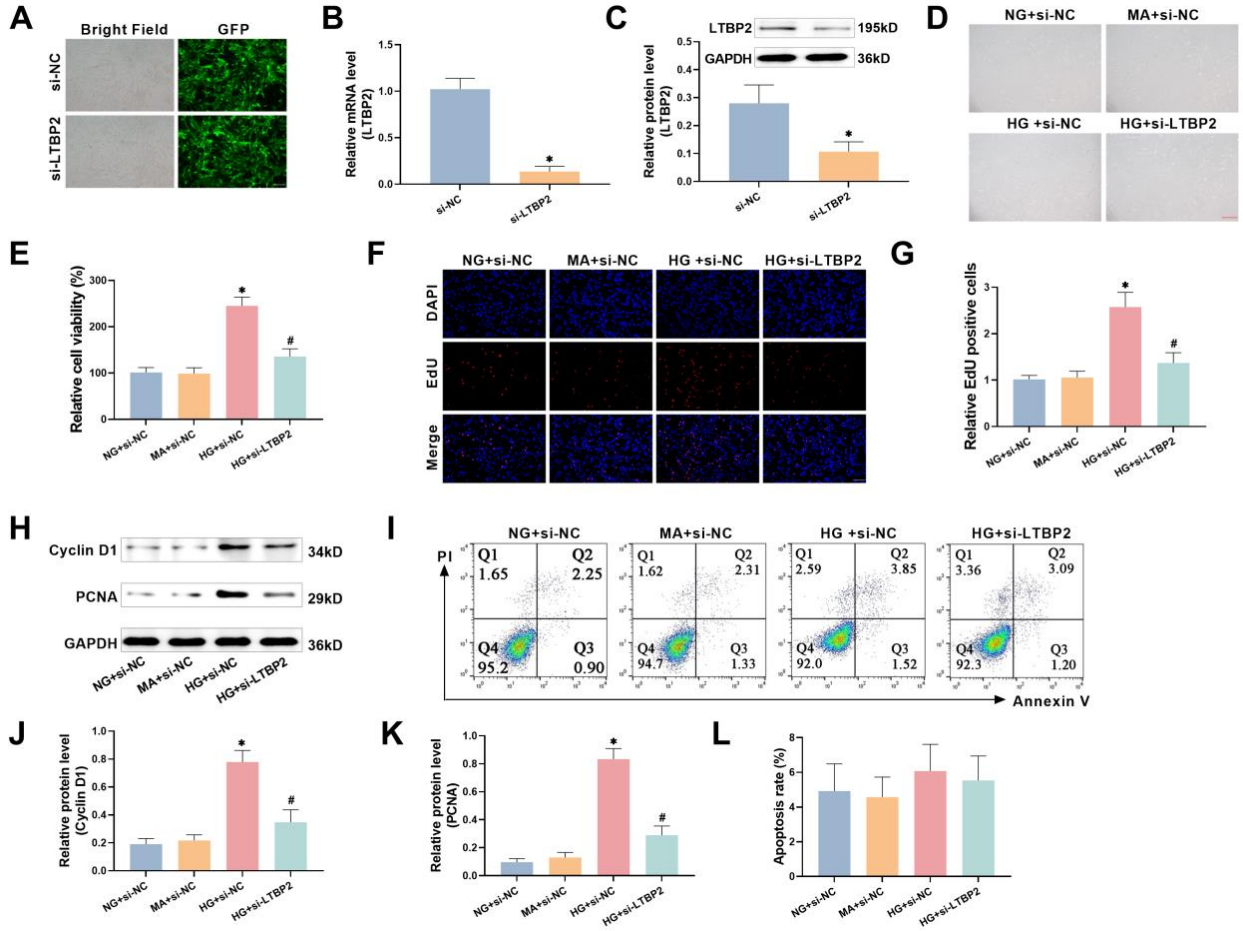

Figure 2. Silencing latent transforming growth factor-β binding protein-2 (LTBP2) attenuates proliferation in HBZY-1 cells
(A) The transfection efficiency was assessed using the green fluorescent protein (GFP) method. (B-C) Examining LTBP2 level through qRT-PCR and Western blot, revealed a decrease in LTBP2 expression after transfection with si-LTBP2. (D) The survival of cells was observed under a light microscope. (E) The cell viability after transfection with si-NC and si-LTBP2 was monitored by cell counting kit-8 (CCK-8) assay. (F-G) The cell proliferation was identified using EdU staining, and quantified the EdU-positive cells. (H) Examining the level of proliferative proteins Cyclin D1 (J) and proliferating cell nuclear antigen (PCNA) (K) in HBZY-1 cells of different treatment groups through Western blot. (I and L) The apoptosis rate was examined using the Annexin V/PI method. n=3. (*p<0.05 vs NG+si-NC&MA+si-NC, #p<0.05 vs HG+si-NC).

Silencing of LTBP2 Attenuates Fibrosis and Inflammation in HBZY-1 Cells

We explored the effects of LTBP2 on fibrosis and inflammation. After treatment with high glucose, Western blot analysis revealed that fibrosis-associated proteins FN, Col Ⅰ, and Col IV levels were notably elevated, whereas transfection of si-LTBP2 reduced them (Figure 3A-3B). After high glucose treatment, we found that FN, Col Ⅰ, and Col IV levels were markedly elevated as shown by immunofluorescence, whereas silencing LTBP2 reduced the effect of high glucose treatment (Figure 3C-3D). We also observed collagen fiber deposition in HBZY-1 cells by Sirius red staining. The findings indicated that the collagen fibre content in HBZY-1 cells was elevated after high glucose treatment, and transfection with si-LTBP2 caused a significant decline in collagen fibre content (Figures 3E-3F). Additionally, we used ELISA kits to measure intracellular inflammatory factor levels. The result demonstrated that after being treated with high glucose, TNF-α, MCP-1, IL-1β and IL-6 levels showed a notable increase in HBZY-1 cells, and a notable decrease in inflammatory factor levels after transfection with si-LTBP2 (Figure 3G-3J). These findings suggest that

exposure to high glucose led to increased fibrosis and inflammation in HBZY-1 cells, whereas silencing LTBP2 attenuated high glucose-induced cellular fibrosis and inflammation.

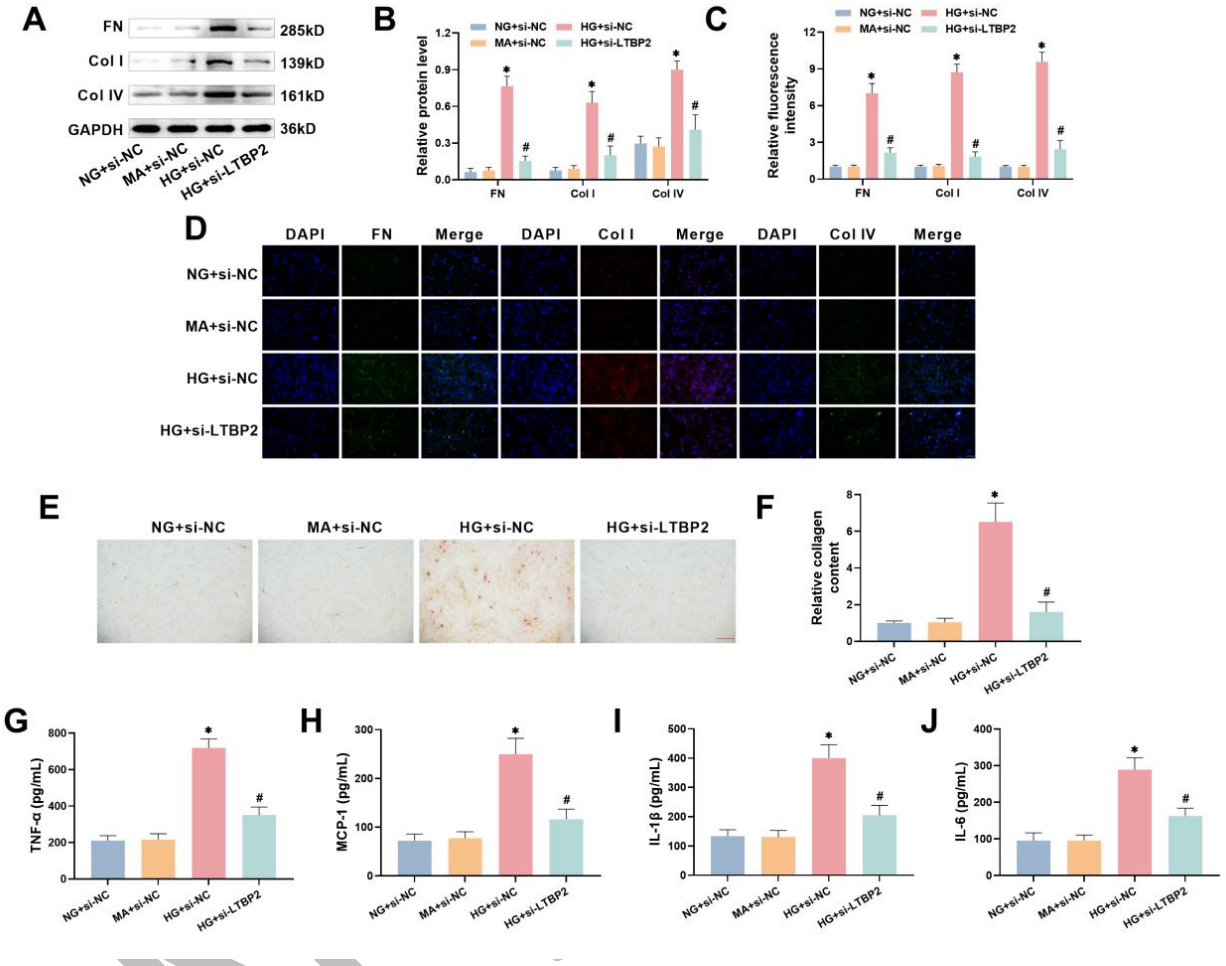

Figure 3. Silencing latent transforming growth factor-β binding protein-2 (LTBP2) attenuates fibrosis and inflammation in HBZY-1 cells.

(A-B) Examining the fibronectin (FN), collagen I (Col Ⅰ) and collagen IV (Col IV) levels through Western blot. (C-D) Immunofluorescence detection of localized expression of FN, Col Ⅰ and Col IV. (E-F) The collagen content was observed by Sirius red staining. (G-J) The cell supernatants from HBZY-1 cells following various treatments were gathered, and tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin-1β (IL-1β), and interleukin-6 (IL-6) levels were assessed using ELISA Kits. n=3. (*p<0.05 vs NG+si-NC&MA+si-NC, #p<0.05 vs HG+si-NC).

Silencing of LTBP2 Inhibits HG-induced PI3K/Akt/NF-κB Pathway Activation in HBZY-1 Cells

To explore the mechanisms by which LTBP2 reduces cell proliferation, fibrosis, and inflammation, we evaluated the relevant signaling pathways. Western blot findings revealed that the phosphorylation of PI3K, Akt, and p65 was notably increased in HBZY-1 cells after high glucose treatment, whereas silencing LTBP2 attenuated this effect. Notably, the PI3K/Akt activator 740 Y-P intervention attenuated the effects of silencing LTBP2, resulting in a notable rise in PI3K, Akt, and p65 phosphorylation. In contrast, the NF-κB activator PMA intervention significantly increased p65

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phosphorylation, without significantly affecting PI3K and Akt, suggesting that NF-κB activation depends on PI3K/Akt (Figures 4A-4D). These results confirm that high glucose treatment activated the PI3K/Akt/NF-κB pathway, while silencing LTBP2 inhibits the activation of this pathway.

Silencing of LTBP2 Attenuates HG-induced Proliferation of HBZY-1 cells by Hindering the PI3K/Akt/NF-κB Pathway

To determine whether silencing LTBP2 inhibits cell proliferation by hindering the PI3K/Akt/NF-κB pathway, we treated HBZY-1 cells with PI3K/Akt and NF-κB activators and assessed cell viability using the CCK-8 assay. The findings indicated that silencing LTBP2 ameliorated high glucose-induced cell proliferation, whereas 740 Y-P and PMA interventions attenuated the effect of silencing LTBP2 (Figures 5A-5B). In addition, silencing LTBP2 significantly reduced the number of EdU-positive HBZY-1 cells, while treatment with 740 Y-P and PMA notably increased the amount of EdU-positive cells (Figure 5C-5D). Western blot results also revealed that silencing LTBP2 led to a significant decline in Cyclin D1 and PCNA levels in HBZY-1 cells, whereas both 740 Y-P and PMA interventions partially reversed the effects of silencing LTBP2 (Figures 5E-5G). These results indicate that the suppressive effect of silencing LTBP2 on high glucose-induced cell proliferation was diminished by the 740 Y-P and PMA interventions, suggesting that LTBP2 plays a role in regulating the PI3K/Akt/NF-κB pathway.

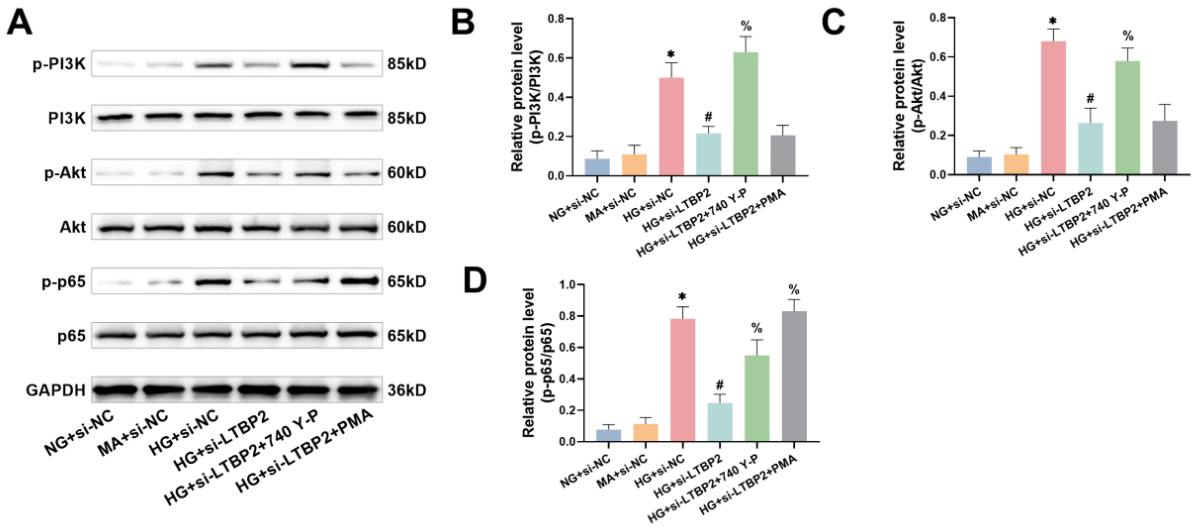

Figure 4. Silencing latent transforming growth factor-β binding protein-2 (LTBP2) inhibits HG-induced phosphoinositol-3 kinase (PI3K)/protein kinase B (Akt)/nuclear factor kappa-B (NF-κB) pathway activation in HBZY-1 cells.
(A) The PI3K/Akt activator 740 Y-P or the NF-κB activator PMA was added 30 min before modelling in the HG group, and PI3K (B), Akt (C), and P65 (D) phosphorylation levels were detected through Western blot. n=3. (*p<0.05 vs NG+si-NC&MA+si-NC, #p<0.05 vs HG+si-NC, %p<0.05 vs HG+si-LTBP2).

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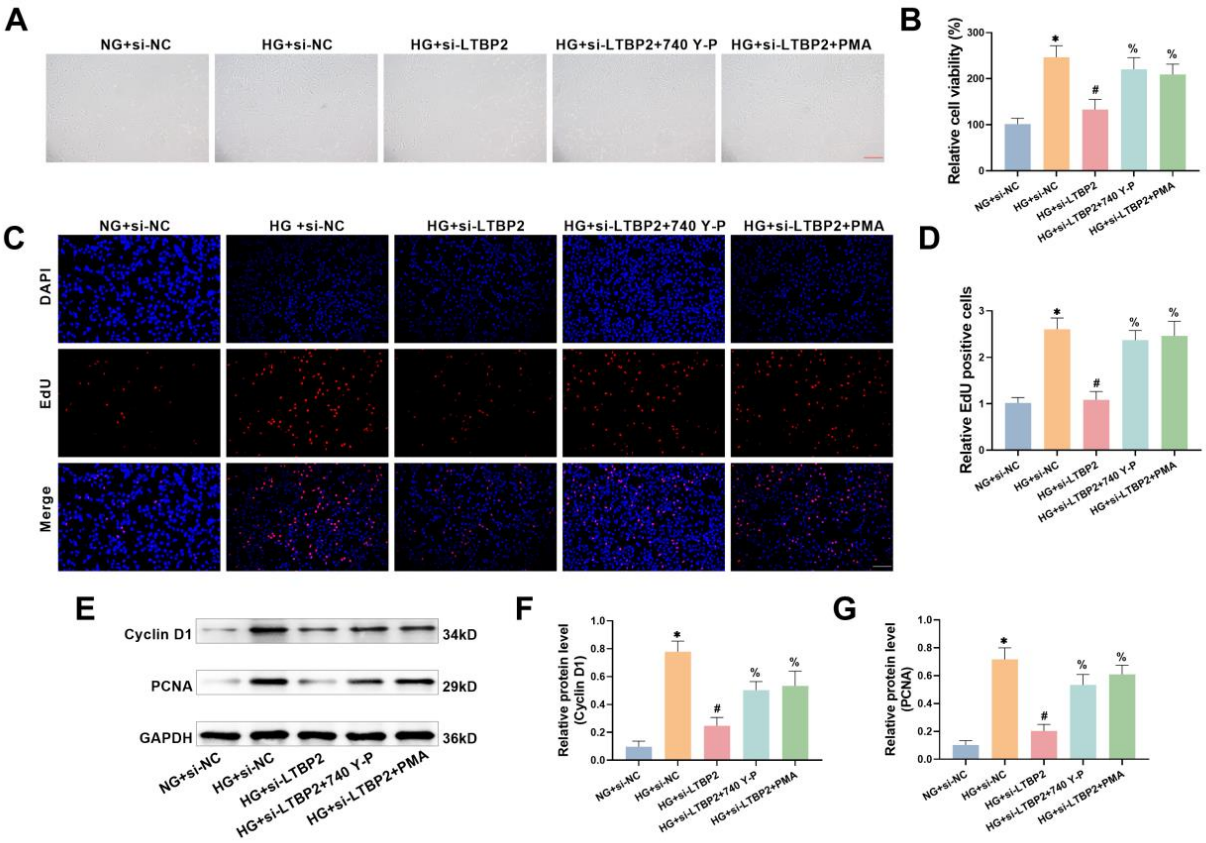

Figure 5. Silencing latent transforming growth factor-β binding protein-2 (LTBP2) attenuates proliferation in HBZY-1 cells by hindering the phosphoinositol-3 kinase (PI3K)/protein kinase B (Akt)/nuclear factor kappa-B (NF-κB) pathway.
(A)The HBZY-1 cell survival in various treatment groups was monitored using a light microscope. (B) Utilizing cell counting kit-8 (CCK-8) assay monitored the cell viability. (C-D) The cell proliferation was identified using EdU staining and quantified the EdU-positive cells. (E-G) Western blot examined proliferative proteins Cyclin D1 (F) and proliferating cell nuclear antigen (PCNA) (G) levels in different treatment groups. n=3. (*p<0.05 vs NG+si-NC&MA+si-NC, #p<0.05 vs HG+si-NC, %p<0.05 vs HG+si-LTBP2).

Silencing of LTBP2 Attenuates HG-induced Fibrosis and Inflammation in HBZY-1 Cells by Inhibiting the PI3K/Akt/NF-κB Pathway

Finally, we explored whether LTBP2 suppresses cellular fibrosis and inflammation by inhibiting the PI3K/Akt/NF-κB pathway. After silencing LTBP2, the FN, Col I and Col IV levels in HBZY-1 cells were significantly decreased. However, both 740 Y-P and PMA interventions reversed the inhibitory effect of silencing LTBP2 on fibrosis in HBZY-1 cells (Figure 6A-6D). By Sirius red staining, we observed that silencing LTBP2 significantly reduced collagen fiber content in HBZY-1 cells, and this effect was reversed by both 740 Y-P and PMA interventions (Figures 6E-6F). In addition, silencing LTBP2 significantly decreased the secretion of TNF-α, MCP-1, IL-1β, and IL-6, but 740 Y-P and PMA interventions partially reversed the effects of silencing LTBP2 (Figures 6G-6J). These results further suggest that silencing LTBP2 can alleviate cellular fibrosis and inflammation induced by high glucose by inhibiting the PI3K/Akt/NF-κB pathway.

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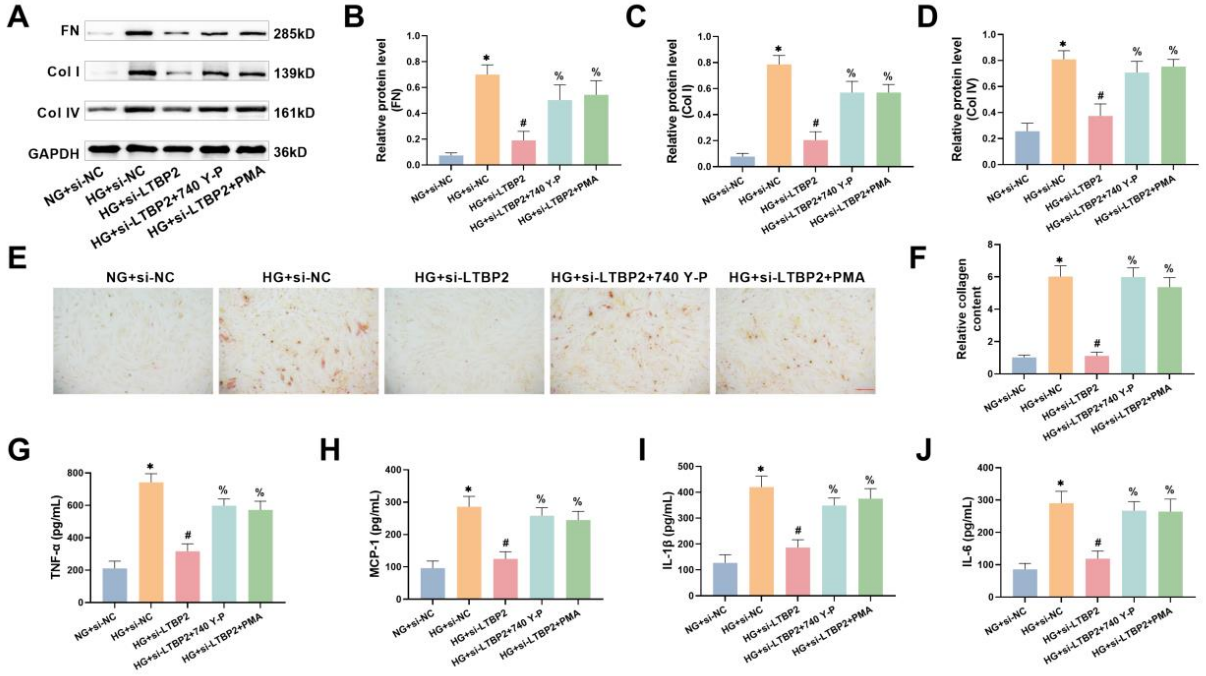

Figure 6. Silencing latent transforming growth factor-β binding protein-2 (LTBP2) attenuates high glucose (HG)-induced fibrosis and inflammation in HBZY-1 cells by inhibiting the phosphoinositol-3 kinase (PI3K)/protein kinase B (Akt)/nuclear factor kappa-B (NF-κB) pathway.

(A-D) Examining the fibronectin (FN) (B), collagen I (Col I) (C), and collagen IV (Col IV) (D) levels through Western blot. (E-F) The collagen content was observed by Sirius red staining. (G-J) The cell supernatants from HBZY-1 cells following various treatments were gathered, and tumor necrosis factor-α (TNF-α), Monocyte chemoattractant protein-1 (MCP-1), interleukin-1β (IL-1β), and interleukin-6 (IL-6) levels were assessed utilizing ELISA Kits. n=3. (*p<0.05 vs NG+si-NC&MA+si-NC, #p<0.05 vs HG+si-NC, %p<0.05 vs HG+si-LTBP2).

DISCUSSION

The complex nature of the etiology and development of DN remain incompletely understood. Numerous studies have demonstrated that DN is the result of a combination of genetic, environmental, and dietary factors that disrupt sugar, protein and lipid metabolism, leading to changes in the intra- and extracellular environments and activation of pathways under hyperglycemic conditions.[26,27] High glucose-induced hyperproliferation and hypertrophy of glomerular mesangial cells caused by high glucose promotes the progression of DN.[28] Consequently, HBZY-1 cells treated with high glucose serve as a classical *in vitro* model to mimic DN.[29] Our research indicated that high glucose treatment caused a significant increase in HBZY-1 cell viability and proliferation, consistent with the previous findings.[30] Furthemore, after high glucose treatment, LTBP2 expression in HBZY-1 cells was markedly increased, and the longer the high glucose treatment time, the higher the LTBP2 expression level. Moreover, silencing LTBP2 attenuated the abnormal proliferation of HBZY-1 cells following high glucose treatment, suggesting that LTBP2 may be involved in regulating DN progression.

The pathogenesis of DN is extremely complex. Hyperglycemia and lipid metabolism disorders are the initiating factors for the development of diabetic nephropathy, and hyperglycemia stimulates metabolic and hemodynamic disorders, induces the release of pro-inflammatory and pro-fibrotic factors, and leads to chronic inflammatory infiltration, oxidative stress, and insulin resistance.[31,32] MCP-1, a key mediator of innate immunity and inflammation, can stimulate an inflammatory response by recruiting mononuclear macrophages, which in turn triggers renal tubular

atrophy as well as interstitial fibrosis, and is considered an inflammatory marker in DN.[33] Several studies have shown that activated T lymphocytes in the renal interstitium of diabetic patients release high levels of pro-inflammatory factors, such as TNF-α, IL-1β, and IL-6, and further promote inflammation by activating macrophages.[25,34] In addition, inflammation promotes the production of ROS. Under normal circumstances, the cellular antioxidant defense system possesses a strong antioxidant capacity, effectively removing harmful substances and protecting cells. However, under pathological conditions, the excessive release of ROS overwhelms the body's antioxidant defense mechanisms, further contributing to the progression of diabetes mellitus.[35] These pro-inflammatory factors are closely linked to DN progression. Our findings revealed TNF-α, MCP-1, IL-1β and IL-6 levels significantly increased in HBZY-1 cells treated with high glucose, whereas silencing LTBP2 ameliorated inflammation, confirming LTBP2 is involved in regulating the inflammation of DN.

The ECM is an important component of tissues and organs, consisting of collagen, fibronectin, proteoglycans, and glycoproteins, which provide structural support for tissues and cells.[36,37] Renal fibrosis, a prevalent pathological process in DN, is characterized by excessive deposition of ECM, with the underlying etiology being the gradual loss of the kidney's ability to repair itself due to persistent inflammation and tissue damage.[32,38] In this study, after exposure to high glucose, we observed that FN, Col I, and Col IV levels were significantly elevated, along with elevated levels of collagen fibers, suggesting that high glucose promoted cell fibrosis. Transfection with si-LTBP2 reduced the high glucose-induced fibrosis, in combination with the above findings, further confirmed that LTBP2 can modulate inflammation and fibrosis progression in DN.

Numerous studies have indicated that targeting the PI3K/Akt pathway could alleviate the fibrotic process in a variety of diseases, including DN.[39-41] Wang et al. found that the up-regulation of LTBP2 level is linked to the PI3K/Akt/mTOR pathway activation[42]. Furthermore, the NF-κB pathway mediates the fibrotic process and inflammatory response in type 2 DN.[43] Zou et al. revealed that overexpression of LTBP2 induces differentiation of fibroblasts through the NF-κB pathway, demonstrating that LTBP2 has a crucial impact on the process of pulmonary fibrosis.[44] It is thus evident that LTBP2-regulated inflammatory response and fibrotic process in DN may be closely related to the PI3K/Akt and NF-κB pathways. In our research, high glucose treatment promoted the phosphorylation of PI3K/Akt/NF-κB pathway-associated proteins. However, silencing LTBP2 could inhibit the PI3K/Akt/NF-κB pathway, which may be an important mechanism for its amelioration of DN. Subsequently, we used PI3K/Akt and NF-κB activators to treat in HBZY-1 cells and found that the activators attenuated the effects of silencing LTBP2 and promoted high glucose-induced aberrant cell proliferation, fibrosis, and inflammation, confirming that silencing LTBP2 ameliorates DN caused by high glucose through inhibiting the PI3K/Akt/NF-κB pathway.

In summary, in our research demonstrates, high glucose treatment upregulated LTBP2 expression in HBZY-1 cells. Notably, LTBP2 activates the PI3K/Akt/NF-κB pathway and therby promoting proliferation, fibrosis, and inflammation in HBZY-1 cells (Figure 7).

Following treatment with high glucose, LTBP2 exhibits a high expression level in HBZY-1 cells, and the PI3K/Akt/NF-κB pathway was activated. Silencing of LTBP2 attenuated cell proliferation, fibrosis, and inflammation and inhibited this pathway. Additionally, activators of the PI3K/Akt/NF-κB pathway attenuated the effects of LTBP2 silencing, suggesting that LTBP2 silencing exerts its effects by inhibiting this pathway. Based on the findings mentioned above, we hypothesize that the high level of LTBP2 and the PI3K/Akt/NF-κB pathway activation may play crucial roles in DN progression, which provides a reliable reference for therapeutic targets and drug screening in DN. However this study has some limitations, and the effects of LTBP2 in animal models warrant further investigation in the future.

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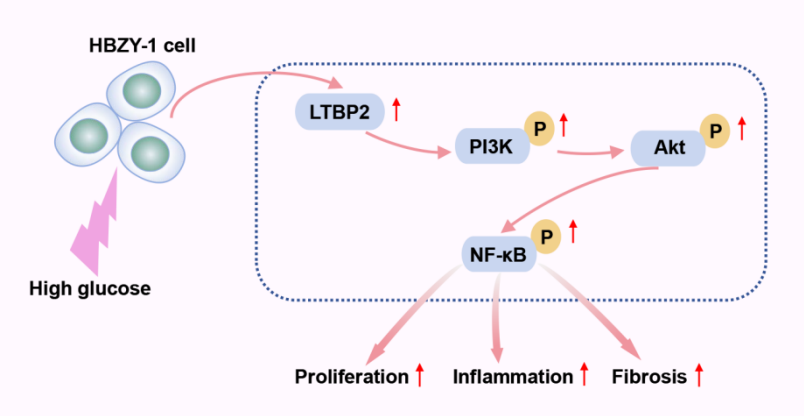

Figure 7. Schematic mechanism of action.
High glucose treatment upregulated latent transforming growth factor-β binding protein-2 (LTBP2) levels in HBZY-1 cells and promoted proliferation, fibrosis, and inflammation by activating the phosphoinositol-3 kinase (PI3K)/protein kinase B (Akt)/nuclear factor kappa-B (NF-κB) pathway.

STATEMENT OF ETHICS

Not applicable.

FUNDING

This work was supported by Wuhan Municipal Health Commission in 2021 (No. WX21B31).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

Not applicable.

DATA AVAILABILITY

The data supporting the findings of this study can be obtained from the corresponding author, Dan Luo, upon request.

AI ASSISTANCE DISCLOSURE

Not applicable.

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