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YY1: A novel therapeutic target for diabetic nephropathy orchestrated renal fibrosis

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ABSTRACT

Background: Renal fibrosis promotes the development of diabetic nephropathy (DN). A growing number of studies have reported that Yin Yang 1 (YY1), which is involved in cellular proliferation and differentiation, plays a crucial role in the pathogenesis of many diseases, such as pulmonary fibrosis, hepatic steatosis and cancer.
Methods: We detected the expression of YY1 under various glucose concentration and time gradient conditions. Rapamycin was used to verify the mTORC1/p70S6K/YY1 signaling pathway in HK-2 cells. We used *db*/*db* mice to examine the connection between renal fibrosis and YY1. A luciferase assay and chromatin immunoprecipitation (ChIP) assay were used to identify whether YY1 directly regulated α-SMA by binding to the α-SMA promoter. RNA silencing and overexpression were performed by using a YY1 expression/knockdown plasmid to investigate the function of YY1 in renal fibrosis of DN.
Results: YY1 expression and subsequent nuclear translocation were upregulated in a glucose- and time-dependent manner via the mTORC1/p70S6K signaling pathway in HK-2 cells. YY1 expression and nuclear translocation was significantly upregulated in *db*/*db* mice. Furthermore, YY1 upregulated α-SMA expression and activity in high-glucose-cultured HK-2 cells. Overexpression of YY1 promoted renal fibrosis in *db*/*m* mice mainly by upregulating α-SMA expression and inducing epithelial-mesenchymal transition (EMT) in vitro and in vivo. Finally, downregulation of YY1 reversed renal fibrosis by improving EMT in vivo and in vitro.
Conclusions: These results reveal that upregulation of YY1 plays a critical role in HG-induced deregulation of EMT-associated protein expression, which finally results in renal fibrosis of DN. Therefore, decreasing YY1 expression might represent a new therapeutic target for diabetic nephropathy-induced renal fibrosis.

1. Introduction

Known as diabetic glomerulosclerosis, diabetic nephropathy (DN) is a common microvascular complication of type 1 and type 2 diabetes, and its incidence increases with the duration of diabetes, leading to chronic renal failure and end-stage renal disease (ESRD) [1]. Based on the complex pathological mechanism of DN, current treatment strategies mainly aimed at treating the symptoms, include reducing blood glucose levels and blood pressure, correcting abnormal metabolic lipids, improving insulin sensitivity and reducing proteinuria (by using angiotensin-converting enzyme inhibitors and angiotensin receptor blockers) [2,3]. However, a single treatment strategy cannot completely improve the symptoms of diabetic nephropathy. Although a growing number of studies have pointed out that various factors are involved in the initiation and progression of DN, the molecular determinants of renal fibrosis remain largely unknown due to its sophisticated mechanism. Consistent with the development of fibrosis in most chronic kidney disease, renal fibrosis is an irreversible characteristic of DN, particularly notable are the pathological changes of the glomerulus and tubulointerstitium [4,5]. Studies have shown that glomerular fibrosis appears early in the progression from emergence to overt nephropathy, while tubulointerstitial fibrosis can be obverted in advanced stages and is closely related to the functional injury in the kidney [6–10]. The accumulation of extracellular matrix (ECM) proteins in the mesangial interstitial space is characterized by upregulated collagen proteins and fibronectin, which mainly contribute to the fibrosis in DN [11,12]. Currently, increasing attention has been paid to the epithelial-mesenchymal transition (EMT) of renal tubular epithelial cells in diabetes-induced renal fibrosis, which is characterized by

Abbreviations: YY1, Yin Yang 1; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; α-SMA, α-smooth muscle actin; EMT, epithelial-mesenchymal transition; BUN, blood urea nitrogen; Cr, creatinine; DN, Diabetic nephropathy; FBG, Fasting blood glucose; HK-2, Human proximal tubular epithelial cells; HG, high glucose; NG, normal glucose.

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the loss of epithelial proteins and the acquisition of mesenchymal proteins [13]. The formation of EMT aggravates the accumulation of ECM proteins but can be attenuated by various inducers, such as cytokines, high glucose (HG) and advanced glycation end products (AGEs) [14–16]. Nevertheless, the molecular mechanisms of the process of renal tubular EMT underlying the complexity of renal fibrosis in DN remain poorly understood.

With in-depth research, a growing number of studies have pointed out various profibrotic mediators associated with the progression of DN renal fibrosis, such as transforming growth factor-β (TGF-β), connective tissue growth factor, angiotensin II, endothelin-1, fibroblast growth factor and so on [17–23]. New studies have shown that Yin Yang 1 (YY1) plays an important role in cellular proliferation, migration and invasion and that YY1 has also been shown to act as a novel transcription factor involved in hepatic triglyceride metabolism in obesity [24–26]. Furthermore, studies have increasingly found that YY1 is involved in multiple metabolic diseases, such as non-alcoholic fatty liver disease (NAFLD), obesity and diabetes [27–29]. Additional research has shown that YY1 is a novel regulator of fibrosis in other conditions, such as cancer, chronic kidney failure and pulmonary fibrosis [30–32]. Therefore, we investigated the potential role of YY1 in the initiation of renal fibrosis in DN.

In the present study, our data demonstrate that YY1 is a key regulator in the renal fibrosis of DN. We found that HG induces the overexpression and nuclear translocation of YY1 via the mTORC1/P70S6K signaling pathway and activates and upregulates α-SMA, causing EMT of tubular epithelial cells. Thus, YY1 could be applied to prevent or ameliorate diabetic nephropathy-induced renal fibrosis.

2. Experimental procedure

2.1. Materials

For biochemical measurements, fasting blood glucose (FBG), creatinine (Cr) and blood urea nitrogen (BUN) reagent kits were obtained from Jiancheng Bioengineering Institute (Nanjing, China). A urinary albumin assay kit was purchased from Mlbio (Shanghai, China). Primary antibodies for mTOR (2983), p-mTOR (Ser2448) (5536), p70S6K (2708), p-p70S6K (Thr389) (9423), E-cadherin (3195), ZO-1 (8193), vimentin (5741), and snail (3879) were from Cell Signaling Technology (MA, USA). Antibodies against YY1 (ab109228), twist (ab175430), α-SMA (ab32575), Lamin B (ab133741), laminin (ab11575) and collagen IV (ab6586) were from Abcam (CA, USA). Antibodies against β-actin (AP0060) were from Bioworld (St. Louis, USA). Dylight 594 donkey anti-rabbit antibody was purchased from EarthOx Life Sciences (CA, USA).

2.2. Animals

Male C57BLKS/J background Lep^{db}/Lep^{db} (*db*/*db*) (7 weeks old, 30–35 g) and the nondiabetic control Lep^{db}/m (*db*/*m*) littermate mice (7 weeks old, 15–20 g) were purchased from the Model Animal Research Center of Nanjing University (Jiangsu, China). All animals were housed in a barrier environment and provided food and water ad libitum before and during the experiments. All experimental procedures were approved by the animal ethics committee of Xuzhou Medical University. Some *db*/*m* and *db*/*db* mice were fed up to 16 weeks old, and blood, urine and kidney tissues were collected to observe the expression of YY1 in the kidneys of diabetic nephropathy mice.

To observe the effect of mTORC1, *db*/*db* mice were randomly divided into control and treatment groups, which received intraperitoneal injections of vehicle (5% DMSO, *db*/*db* group) and rapamycin (1 mg/kg dissolved in 5% DMSO, *db*/*db* + Rap group), respectively. The *db*/*m* mice also received intraperitoneal injections of 5% DMSO (*db*/*m* group). The three groups of mice were treated 3 times a week for 8 weeks.

In order to explore the effect of YY1 on diabetic nephropathy, *db*/*m* mice were randomly divided into three groups after adaptation for a week: the 0.9% normal saline-injected group (*db*/*m* + NS), the empty lentivirus-injected group (*db*/*m* + LV-NC) and the YY1 overexpression lentivirus-injected group (*db*/*m* + LV-YY1). At the age of 12 weeks, *db*/*db* mice were injected with 0.9% normal saline (*db*/*db* + NS), empty lentivirus (*db*/*db* + LV-NC) or YY1-shRNA lentivirus (*db*/*db* + LV-YY1-shRNA). Mice were treated with lentivirus (6×10^7 TU/mouse) or 0.9% normal saline via tail vein injection. Lentiviruses expressing YY1 (LV-YY1) and YY1 shRNA (LV-YY1-shRNA) were constructed by GenePharma (Suzhou, China). After 1 month, the mice were placed in metabolic cages for urine collection, and the blood samples were also collected. Part of the kidney tissue was fixed in 4% paraformaldehyde, while the remaining tissue was stored at −80 °C for biochemical analysis.

2.3. Cell culture and transfection

The human proximal tubular epithelial cell line HK-2 was a kind gift from Nanjing University. The cells were cultured in DMEM containing 5.56 mmol/L D-glucose (normal glucose, NG), supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY) and 1% penicillin-streptomycin in a humidified incubator at 37 °C with 5% CO_2. To induce EMT, the cells were cultured in medium containing 30 mmol/L, 60 mmol/L and 90 mmol/L D-glucose for 72 h or cultured in medium containing 60 mmol/L (high glucose, HG) for 0 h, 8 h, 24 h and 72 h. Mannitol medium containing 5.56 mmol/L glucose and 54.44 mmol/L mannitol was used as the osmotic control (MA). For treatment with rapamycin (Cell Signaling, Beverly, MA, USA), After serum starvation for 24 h, HK-2 cells were treated with HG solution and 20 nmol/L rapamycin for 72 h (HG + Rap).

For YY1 shRNA experiments, HK-2 cells stimulated with HG were transfected with the control lentivirus vector (HG + Vehicle) or YY1 shRNA lentivirus vector (HG + YY1-shRNA) supplemented with 8 μg/ml polybrene for 72 h. HK-2 cells were cultured in HG without other treatments (HG) as a control. For YY1 overexpression experiments, HK-2 cells cultured with NG were transfected with the control lentivirus vector (NG + Vehicle) or YY1 overexpression lentivirus vector (NG + YY1) supplemented with 8 μg/ml polybrene for 72 h, and cells cultured with NG served as controls (NG). The lentiviral vectors for specific targeted human YY1 expression and scrambled control sequences were designed and produced by GeneChem (Shanghai, China), and the shRNA sequences are shown below (Supplemental Table 1).

2.4. Measurement of renal function and biochemical parameters

Renal function was assessed through the measurement of blood urea nitrogen (BUN) and blood creatinine (Cr) and urinary albumin levels. BUN and Cr were determined using assay kits from Jiancheng (Nanjing, China). Urinary microalbumin to creatinin levels (mAlb/Cr) were measured by a competitive ELISA kit from Mlbio (Shanghai, China). Serum levels of fasting blood glucose (FBG) were measured by test strips from LifeScan (CA, USA). The kidney index (mg/g) was calculated as the ratio of the weight of the two kidneys to the body weight of the mice.

2.5. Quantitative RT-PCR

Total RNA was isolated from kidney cortex and HK-2 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and qRT-PCR was performed as described previously [11]. The primers were designed and synthesized at Sangon Biotech (Shanghai, China) (Supplemental Table 1).

2.6. Western blot analysis

Total, nuclear and cytoplasm protein analysis of mice and HK-2 cells was carried out as described previously [11].

2.7. Immunofluorescence staining

The kidney samples were fixed in 4% paraformaldehyde and embedded in paraffin. Then, sections of 5 μm thickness were cut perpendicularly to the long axis of the kidney. HK-2 cells cultured on glass coverslips were washed three times with cold PBS and fixed with cold methanol at −20 °C for 20 min. After three extensive washes with PBS, the samples were blocked with 5% BSA for 1 h at room temperature, incubated with primary antibody for 2 h at room temperature, and then incubated with a secondary antibody conjugated with DyLight 488 or DyLight 594 (Earthox, Millbrae, CA, USA) at 37 °C for 1 h. Subsequently, the samples were stained with DAPI (Beyotime, Nantong, China) to detect the cell nuclei. The coverslips were mounted on glass slides, and the images were viewed with an Olympus BX43F fluorescence microscope (Tokyo, Japan).

2.8. Histology and immunohistochemistry analysis

The kidney samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 5 μm thickness were cut perpendicularly to the long axis of the kidney for immunohistochemistry and morphometric analysis. The sections were examined using an Olympus BX43F microscope. The three most central sections of each sample were analyzed. Linear measurements were obtained using an image analysis system (Image-Pro Plus 4.0, Media Cybernetics, Silver Spring, MD).

2.9. Luciferase assay

RNA silencing and overexpression were performed as described previously. HK-2 cells at 30% confluence were transfected with a mixture of α-SMA-dependent luciferase reporter and Renilla (Promega, USA) using PloyFect. The HK-2 cells were lysed 72 h after transfection, and luciferase activity was measured using a dual-luciferase reporter assay system (Promega, USA) following the manufacturer's instructions.

2.10. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with a Magnetic ChIP Kit (P-2026, Epigentek, USA) according to the manufacturer's instructions. The cells were lysed, and the chromatin was mainly fragmented to 200 bp to 1000 bp by sonication shearing. DNA/protein complexes were precipitated by incubation with 0.8 g of antibodies against YY1 or IgG and then incubated with protein A/G magnetic beads for 2 h. After reversal of protein-DNA cross-linking, the DNA was purified, and the abundance of the YY1 and α-SMA promoters was analyzed by qRT-PCR.

2.11. Transmission electron microscopy

For transmission electron microscopy, the pretreatment methods for the kidney samples were performed as described in a previous study [11].The Tecnai G2 TEM (FEI, Hillsboro, OR, USA) was used for viewing and photographing samples.

2.12. Statistical analysis

Statistical was analyses were performed with SPSS 16.0 software. All experimental data were represented as the mean ± SD and compared by using an independent-samples *t*-test or ANOVA followed by the least significant difference (LSD) post hoc test. $P \leq 0.05$ was accepted as statistically significant.

3. Results

3.1. The expression and distribution of YY1 is regulated by HG in HK-2 cells

After serum starvation for 24 h, HK-2 cells were treated with HG medium (60 mmol/L D-glucose) for 0 h, 8 h, 24 h and 72 h. As shown in Fig. 1A, Total YY1 expression was upregulated in a time-dependent manner, as demonstrated by Western blot and immunofluorescence. At the same time, HG induced the redistribution of YY1 from the cytoplasmic to the nuclear fraction of cultured cells. These results showed that the nuclear expression of YY1 increased with time, and the cytoplasmic expression of YY1 decreased in a time-dependent manner (Fig. 1B).

Using the same protocol as above, HK-2 cells were exposed to different concentrations of glucose solution (5.56 mmol/L, 30 mmol/L, 60 mmol/L and 90 mmol/L) for 72 h. As presented in Fig. 1C, D, with the increased glucose dose, the expression of total and nuclear YY1 protein gradually increased, and the expression of cytoplasmic YY1 gradually decreased. In order to eliminate the influence of an osmotic change induced by HG, mannitol was used as an osmotic control. The cellular morphology, the expression of the epithelial cell marker E-cadherin, the mesenchymal cell marker α-SMA and the expression of YY1 (total YY1, nuclear YY1 and cytoplasmic YY1) all showed that the osmotic pressure generated by HG did not affect any of the indexes involved in this study (Supplemental Fig. 1). These results suggest that HG promotes the expression and distribution of YY1.

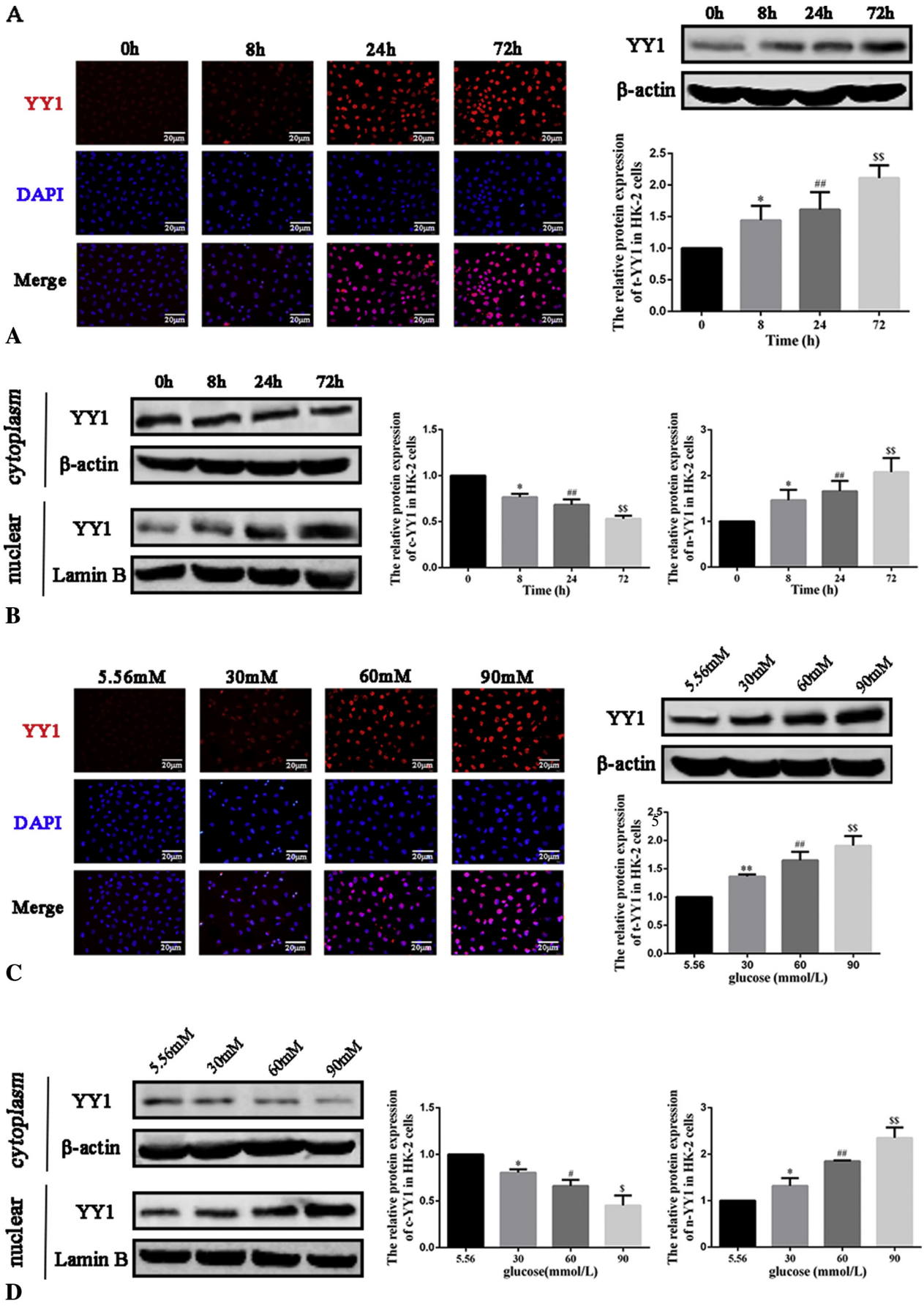
3.2. mTORC1 mediates YY1 expression in HK-2 cells after HG stimulation

A previous study found that the phosphorylation of mTORC1 rose notably, promoting renal fibrosis in diabetic rats [11]. To investigate whether the mTOR pathway is involved in HG-induced YY1 expression in HK-2 cells, we examined the phosphorylation status of mTORC1 and p70S6K protein. As shown in Fig. 2A, B, phosphorylation of mTORC1 at Ser2448 and p70S6K at Thr389 was significantly increased and correlated with time and glucose dose in glucose-stimulated HK-2 cells, suggesting that HG could activate the mTOR pathway of HK-2 cells.

To determine whether mTORC1 mediates YY1 expression, HK-2 cells cultured with HG medium were treated with rapamycin (a specific inhibitor of mTORC1). The results showed that rapamycin significantly decreased the expression of YY1 compared to HG, and reduced the phosphorylation of mTORC1 and p70S6K (Fig. 2C, D). Furthermore, rapamycin reversed HG-induced nuclear translocation of YY1 and promoted redistribution of YY1 from the nucleus to the cytoplasm (Fig. 2E). The levels of all of these factors in the mannitol group were almost identical to those in the NG group, indicating that the osmotic pressure generated by HG did not affect any of the factors assessed. Taken together, these data demonstrate that the mTORC1/P70S6K pathway mediates YY1 expression and its nuclear translocation in HG-cultured HK-2 cells.

3.3. YY1 expression is increased in db/db mice

To explore the effect of HG on YY1 expression in vivo, experiments were carried out in *db*/*db* mouse models. As shown in Supplemental Table 2, creatinine (Cr), blood urea nitrogen (BUN), urinary microalbumin to creatinine (mAlb/Cr) levels and fasting blood glucose (FBG) levels of *db*/*db* mice were markedly elevated compared with those of the *db*/*m* mice. Compared with *db*/*m* mice, the distribution of Masson-positive and Sirius red-positive areas was significantly increased in *db*/*db* mice, indicating collagen accumulation in the renal cortex (Fig. 3A). These results revealed that renal function in diabetic mice was decreased. Furthermore, the expression of total YY1 protein was significantly increased in *db*/*db* mice (Fig. 3B, C), while nuclear YY1 levels increased and cytoplasmic YY1 levels decreased (Fig. 3D). Our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The relative protein expression of t-YY1 in HK-2 cells
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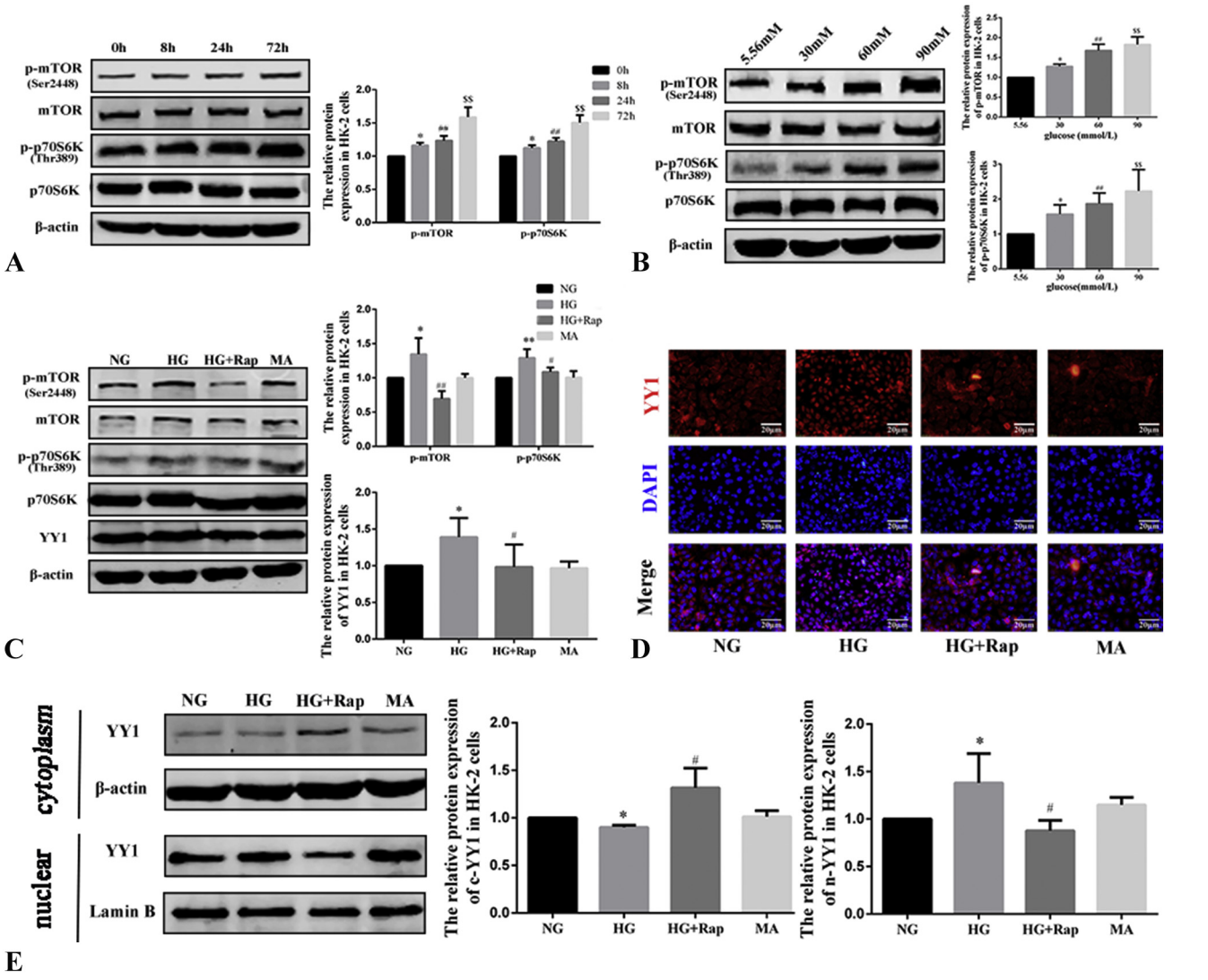

Fig. 2. Effects of HG on mTORC1/p70S6K/YY1 pathway in HK-2 cells. (A) The relative phosphorylation levels of mTORC1 and p70S6K in HK-2 cells exposed to 60 mmol/L glucose for 0 h, 8 h, 24 h and 72 h. (B) The relative phosphorylation levels of mTORC1 and p70S6K in HK-2 cells exposed to 5.56 mmol/L, 30 mmol/L, 60 mmol/L and 90 mmol/L glucose for 72 h. (C,D) The relative protein levels of total YY1, cytoplasm YY1 and nuclear YY1 in HK-2 cells. NG: cells treated with normal glucose 5.56 mmol/L; HG: cells treated with HG 60 mmol/L; HG + Rap: cells treated with HG + 20 nmol/L rapamycin; MA: cells treated with normal glucose and mannitol 24.44 mmol/L. Each bar represents the mean ± SD for groups of three.*$P < 0.05$, **$P < 0.01$, means 8 h vs 0 h, 30 mM vs 5.56 mM or HG vs NG; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, means 24 h vs 0 h, 60 mM vs 5.56 mM or HG + Rap vs HG; $^{\$\$}P < 0.01$, means 72 h vs 0 h or 90 mM vs 5.56 mM.

results indicate that YY1 might be involved in diabetic nephropathy in mice.

3.4. YY1 can directly up-regulate α-SMA in HG-cultured HK-2 cells

It is generally known that α-SMA is a marker of mesenchymal cells. The results of Western blot and immunofluorescence showed that α-SMA expression increased significantly in HK-2 cells treated with 60 mmol/L glucose for 72 h (Fig. 4A), suggesting that HG could stimulate α-SMA expression in HK-2 cells.

To investigate whether YY1 could regulate α-SMA in HK-2 cells, a YY1 overexpression lentiviral vector was used to increase YY1 expression in HK-2 cells (Fig. 4B). The Western blotting and immunofluorescence results showed that overexpression of YY1 in normal glucose (NG)-cultured HK-2 cells resulted in increased expression of α-SMA (Fig. 4C). Further ChIP assays were conducted to confirm the exact mechanism of this phenotype. YY1 directly bound to the α-SMA promoter, and YY1 overexpression increased the amount of binding (Fig. 4D). The luciferase reporter assay showed that the fluorescence intensity of α-SMA was significantly enhanced in the cotransfected YY1 plasmid group when compared with the cotransfected empty vector plasmid group, indicating that YY1 could induce α-SMA promoter activity (Fig. 4E).

In contrast, small interfering RNA was used to decrease YY1 expression in HK-2 cells cultured with HG (Fig. 5A). After silencing of YY1, the expression of α-SMA in HG-cultured HK-2 cells was significantly decreased (Fig. 5B). Similarly, the amount of YY1 binding to the α-SMA promoter and the activity of the α-SMA promoter were both reduced in the YY1-silenced group (Fig. 5C, D). Furthermore, the results showed that transfection of HK-2 cells with control lentivirus vector had no effect on the experimental indicators. These data suggest that YY1 directly regulates α-SMA by binding to the α-SMA promoter.

Fig. 1. Effects of HG on YY1 in HK-2 cells. (A, B) The relative protein levels of total YY1, cytoplasm YY1 and nuclear YY1 in HK-2 cells exposed to 60 mmol/L glucose for 0 h, 8 h, 24 h and 72 h. (C, D) The relative protein levels of total YY1, cytoplasm YY1 and nuclear YY1 in HK-2 cells exposed to 5.56 mmol/L, 30 mmol/L, 60 mmol/L and 90 mmol/L glucose for 72 h. Each bar represents the mean ± SD for groups of three. *$P < 0.05$, **$P < 0.01$, means 8 h vs 0 h or 30 mM vs 5.56 mM; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, means 24 h vs 0 h or 60 mM vs 5.56 mM; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, means 72 h vs 0 h or 90 mM vs 5.56 mM.

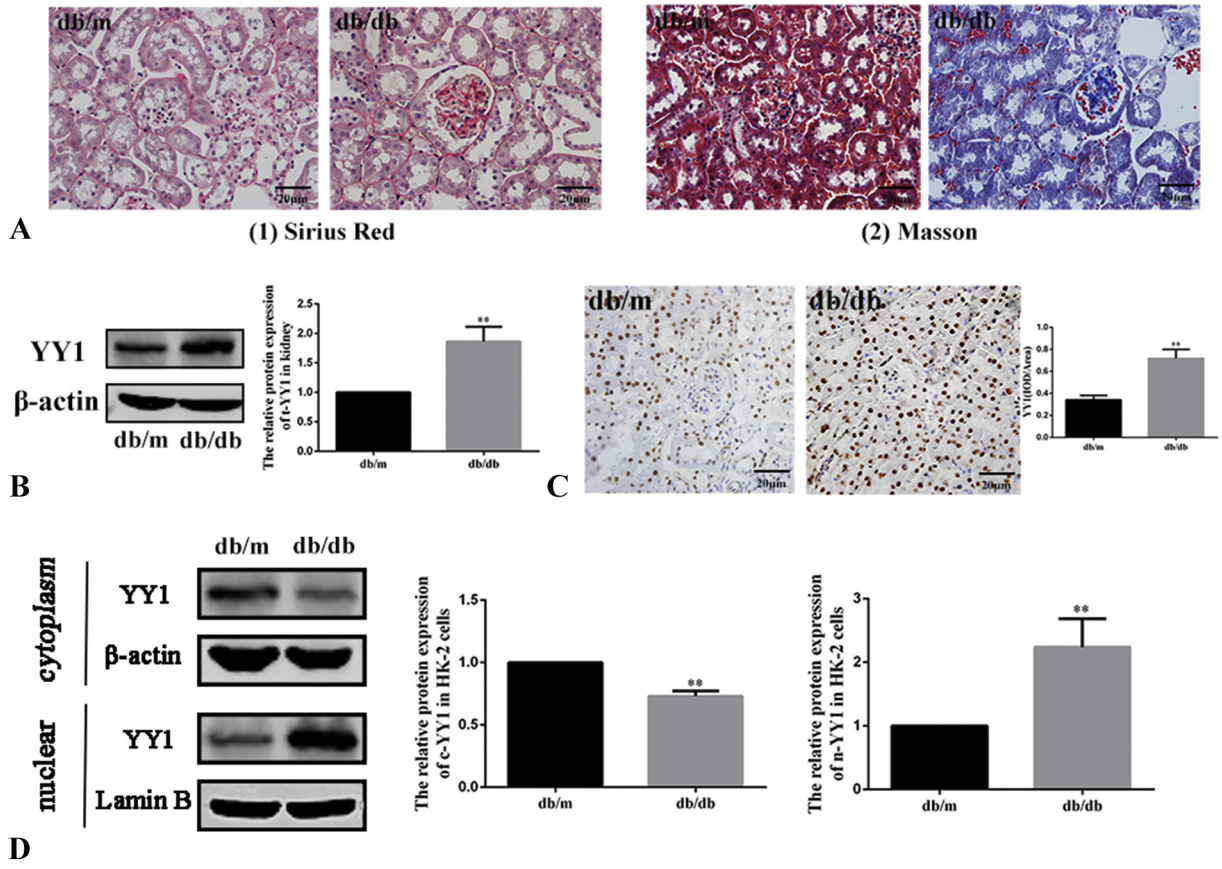

Fig. 3. YY1 expression was increased in *db*/*db* mouse models. (A) Masson staining and Sirius red staining of renal cortex sections of *db*/*m* and *db*/*db* mice. (B) The relative protein levels of total YY1 in mice. (C) The expression of YY1 in mice by immunohistochemistry. (D) The relative protein levels of cytoplasmic and nuclear YY1 in mice. Each bar represents the mean ± SD for groups of six. $^{**}P < 0.01$, *db*/*db* vs *db*/*m* means.

3.5. YY1 overexpression is critical for renal fibrosis in vivo and in vitro

Concerning the involvement of α-SMA in EMT and renal fibrosis and its regulation through YY1, the role of YY1 in the renal fibrosis of DN requires further study. Lentivirus was injected via the tail vein to increase YY1 protein expression in the kidneys of *db*/*m* mice. Western blotting and immunohistochemistry demonstrated that YY1 was observably upregulated in the renal cortex of *db*/*m* mice injected with YY1 lentivirus (Fig. 6A). Compared with those of the mice in the *db*/*m* + NS group, the blood Cr, BUN and urinary mAlb/Cr levels were elevated in YY1 lentivirus-treated mice (Supplemental Table 3), indicating that the renal function of *db*/*m* mice could be significantly reduced by overexpressing of YY1. Increased collagen and ECM accumulation was observed in both the glomerular and renal interstitium of lentiviral-treated *db*/*m* mice as shown by staining with Masson and Sirius red (Fig. 6B) and immunohistochemistry of laminin and type IV collagen (Fig. 6C). In addition, the significantly downregulated expression of epithelial cell markers (E-cadherin and ZO-1) and the upregulated expression of mesenchymal cell markers (α-SMA and vimentin) and transcription factors (snail and twist) were also observed in lentivirus-treated *db*/*m* mice (Fig. 6D). Transmission electron microscopy showed that the increased expression of YY1 in *db*/*m* mice was accompanied by abnormalities in the subcellular structure of renal tubular epithelial cells, including atypical mitochondrial and endoplasmic reticulum expansion (Fig. 6E).

Next, we verified these changes in vitro. HK-2 cells were stimulated with HG solution for different times or with different doses for 72 h. As shown in Fig. S3A, B, the expression of E-cadherin and ZO-1 was suppressed, and the mesenchymal protein expression levels of α-SMA and vimentin, together with the levels of snail and twist transcription factors, increased gradually. These data suggested that HG induced EMT in HK-2 cells in a time- and dose-dependent manner. Furthermore, after YY1 was transfected into HK-2 cells cultured with NG, laminin and IV collagen levels increased (Fig. 6F), while the expression of E-cadherin and ZO-1 was reduced and the expression of α-SMA, vimentin, snail and twist was increased (Fig. 6G). Therefore, our results demonstrate that overexpression of YY1 leads to renal fibrosis in vivo and in vitro.

3.6. YY1 knockdown reverses renal fibrosis in HG-cultured HK-2 cells and db/db mice

After confirming the important role of the increased expression of YY1 protein in fibrosis progression, we probed the effect of knocking down YY1 to alleviate diabetic nephropathy. The expression of YY1 in the renal cortex of *db*/*db* mice was significantly decreased after injection of lentivirus via the tail vein (Fig. 7A). The effect of YY1 on renal fibrosis in *db*/*db* mice was first observed by detecting Cr, BUN and mAlb levels. As shown in Table 1, compared with those of the *db*/*db* + NS mice, the levels of blood Cr, BUN and urinary mAlb/Cr were reduced in the YY1 shRNA lentivirus-treated *db*/*db* mice. Second, Sirius red staining and Masson staining were used to evaluate collagen accumulation (Fig. 7B) and immunohistochemistry of laminin and type IV collagen was conducted to detect ECM (Fig. 7C), showing renal fibrosis progressed in lentivirus-treated *db*/*db* mice compared to *db*/*db* mice injected with normal saline. These results indicated that renal function in diabetic mice was improved by knockdown of YY1. Furthermore, the significantly upregulated expression of epithelial cell markers (E-cadherin and ZO-1) and the downregulated expression of mesenchymal cell markers (vimentin) and transcription factors (snail and twist) were

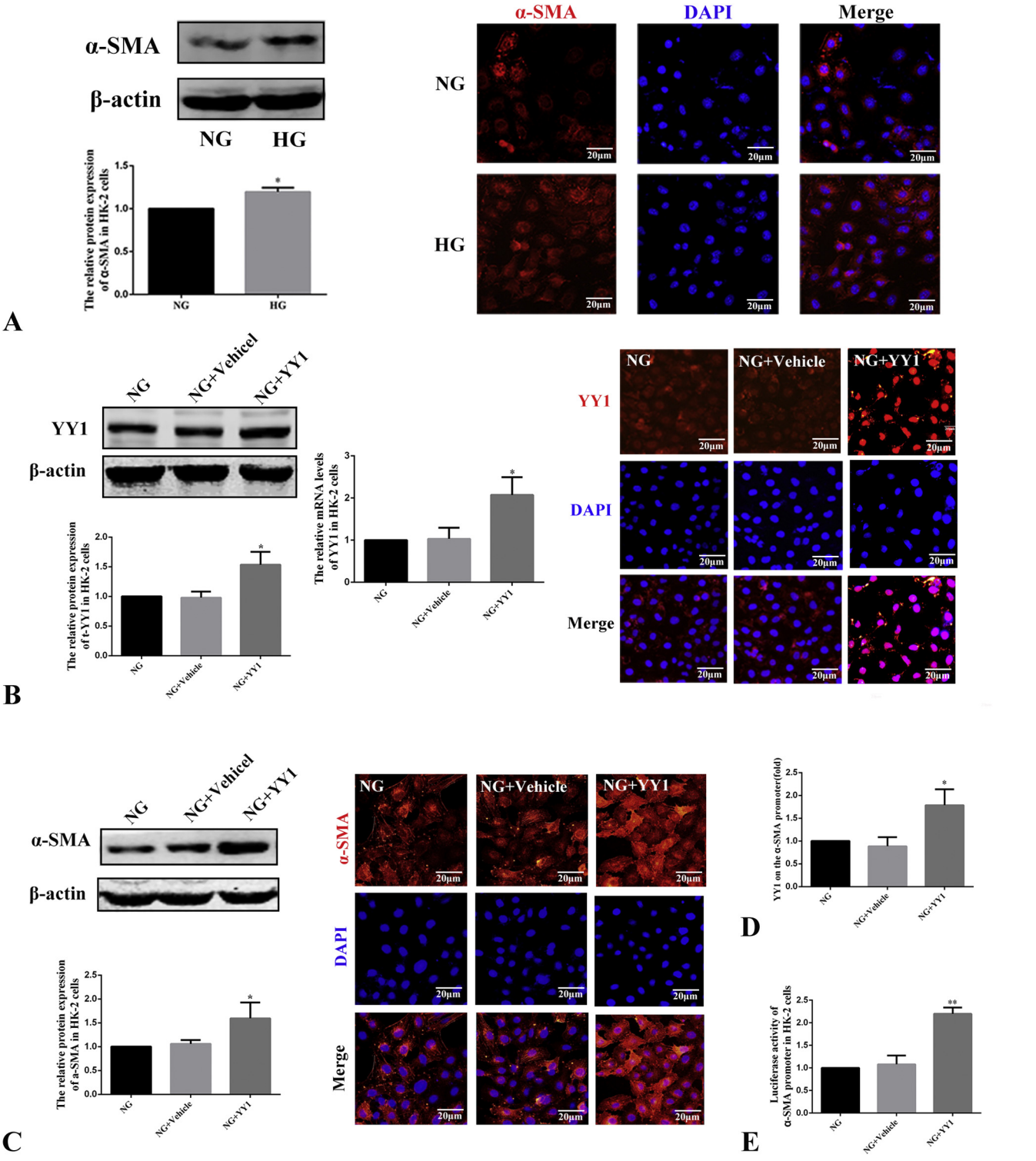

Fig. 4. Effects of YY1 overexpression on α-SMA in HK-2 cells. (A, C) The relative protein levels of α-SMA in HK-2 cells. (B) The relative protein levels and mRNA levels of YY1 in HK-2 cells. (D) The interactions between YY1 and the α-SMA promoter were detected by ChIP. (E) The relative activity of α-SMA in HK-2 cells by luciferase. NG: cells treated with normal glucose 5.56 mmol/L; HG: cells treated with 60 mmol/L glucose; NG + Vehicle: cells treated with NG and transfected with vehicle lentivirus; and NG + YY1: cells treated with NG and transfected with a YY1 overexpression lentivirus. Each bar represents the mean ± SD for groups of three. $^{*}P < 0.05$ and $^{**}P < 0.01$, mean HG vs NG or NG + YY1 vs NG.

also observed in lentivirus-treated *db/db* mice (Fig. 7D). The irregularity of mitochondria in renal tubular epithelial cells was also improved (Fig. 7E).

To further clarify the role of YY1 in the EMT process of DN, we reduced YY1 protein expression in HK-2 cells cultured with HG, and the expression changes of the fibrosis- and EMT-related proteins were consistent with those in vivo (Fig. 7F, G). Taken together, these results indicate that knocking down YY1 effectively improved diabetic nephropathy-induced renal fibrosis by EMT.

4. Discussion

In recent decades, a host of studies has attempted to elucidate the molecular mechanisms of DN so that effective therapies and

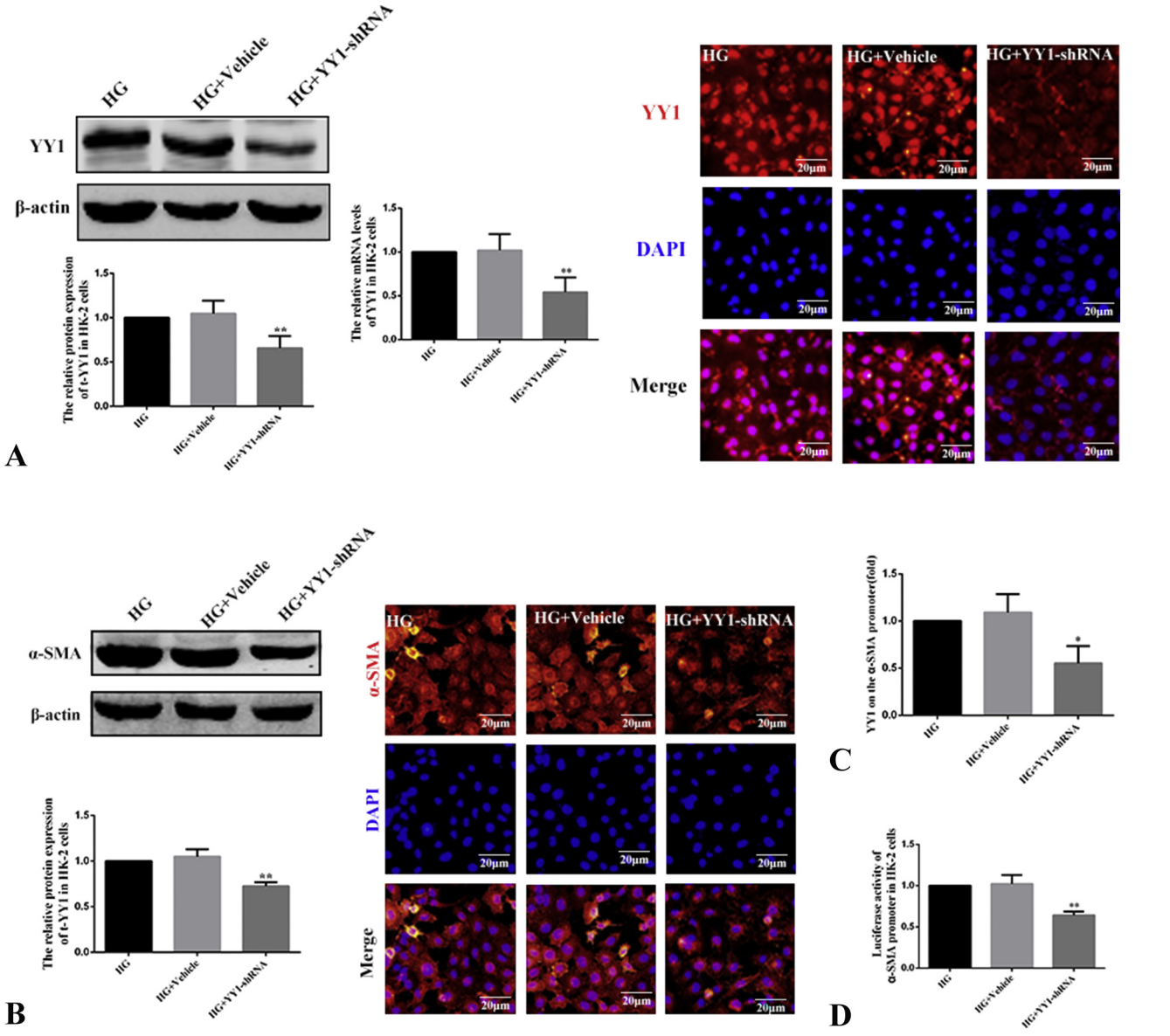

Fig. 5. Effects of YY1 knockdown on α-SMA in HK-2 cells. (A) The relative protein levels and mRNA levels of YY1 in HK-2 cells. (B) The relative protein levels of α-SMA in HK-2 cells. (C) The interactions between YY1 and the α-SMA promoter were detected by ChIP. (D) The relative activity of α-SMA in HK-2 cells by luciferase. HG: cells treated with 60 mmol/L glucose; HG + Vehicle: cells treated with HG and transfected with vehicle lentivirus; and HG + YY1: cells treated with HG and transfected with YY1-shRNA lentivirus. Each bar represents the mean ± SD for groups of three. $^{*}P < 0.05$ and $^{**}P < 0.01$, mean HG + YY1-shRNA vs HG.

preventative strategies can be developed. In the present study, we identified a novel mechanism of renal fibrosis in DN. Our data show for the first time that YY1 is an important regulator of the nuclear transcriptional coactivator α-SMA and EMT-associated proteins in HG-induced DN. In both in vitro HK-2 cells and in vivo diabetic mice, HG significantly induced the expression and nuclear translocation of YY1 via the mTORC1/P70S6K signaling pathway. Furthermore, downregulation of YY1 expression with gene-specific shRNA significantly reduced HG-induced expression and activity of α-SMA and prevented HG-induced EMT, leading to protection against renal fibrosis in DN of *db*/*db* mice.

YY1 is a ubiquitous and multifunctional zinc-finger transcription factor of the polycomb group protein family that can act as a transcriptional repressor, activator or initiator element binding protein and plays an important role in cellular proliferation and differentiation under physiological and disease conditions [33,34]. In addition to being involved in a large number of cancers, previous research found that YY1 is involved in several kidney diseases, such as chronic kidney disease (CKD), drug-induced renal injury, kidney tumor and diabetic nephropathy; further mechanism studies showed that tubular epithelial cell apoptosis, accumulation of p53 and suppression of satellite cell differentiation were closely related to YY1-mediated kidney diseases [35–38]. In addition, it was reported that the expression of YY1 also contributes to the pathogenesis of fibrotic progression of pulmonary tuberculosis, Duchenne muscular dystrophy, lung fibroblasts and so on [39–41]. Our study showed that accompanied by decreased renal function and accumulated collagen in the renal cortex, the expression of YY1 was upregulated in the *db*/*db* mouse model, which indicates that YY1 may play an important and previously underappreciated role in diabetic nephropathy-induced renal fibrosis.

Hyperglycemia, or raised blood sugar, is a major characteristic of diabetes and, over time, leads to serious damage to many of the body's systems, particularly the renal, ocular and nervous systems [42]. In a state of insulin resistance and fasting conditions, hepatic YY1 expression levels were upregulated in mice, and another tubular epithelial cell apoptosis study showed that the translocation of YY1 from the cytoplasm to the nucleus reached peaked at 36 h in HG-treated HK-2 cells [29,38].

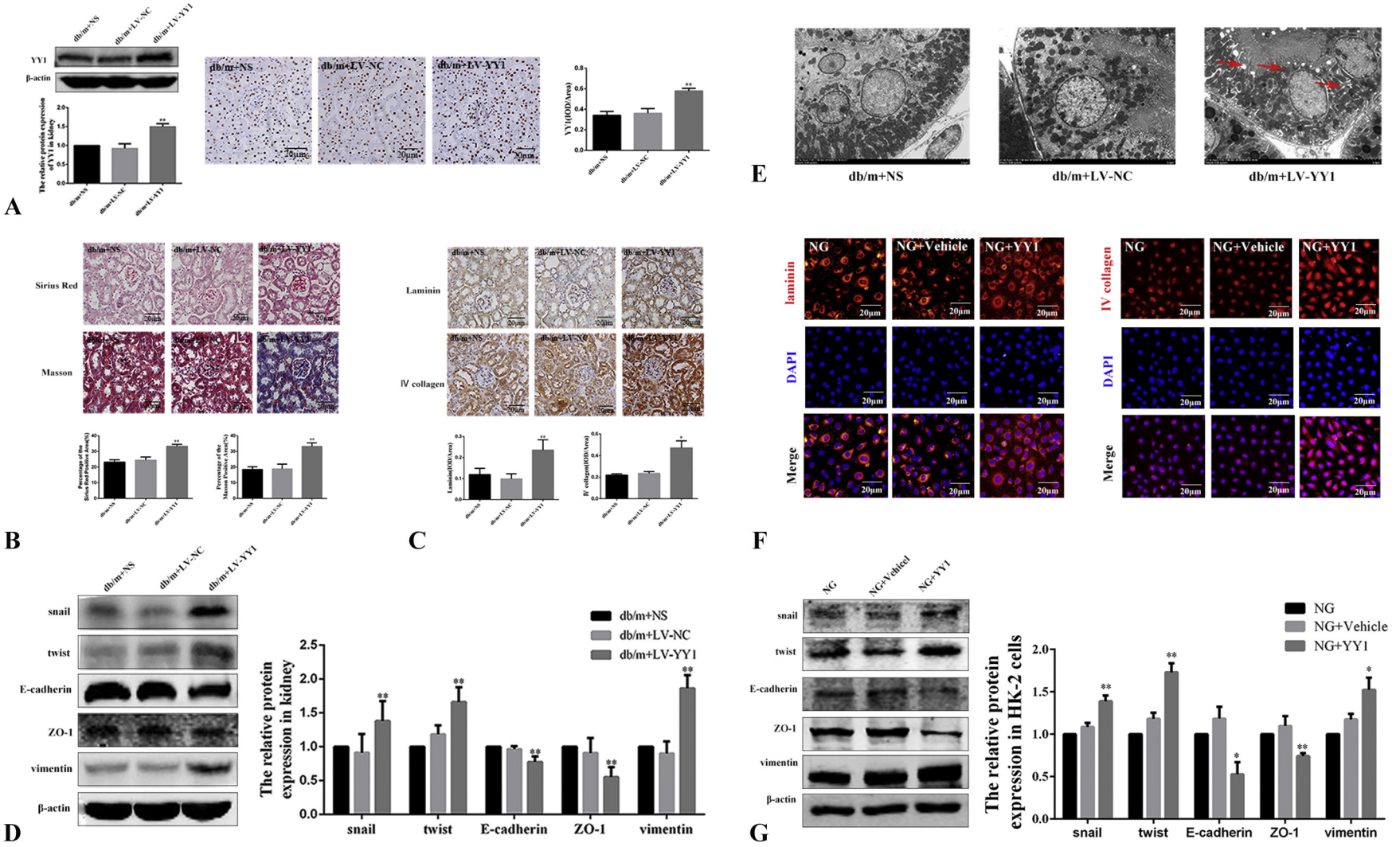

Fig. 6. Effects of YY1 overexpression on renal fibrosis in *db/m* mice and on EMT of HK-2 cells. (A) The relative protein levels of YY1 in mice. (B) Masson staining and Sirius red staining of renal cortex sections of *db/m* mice. (C) The expression of laminin and type IV collagen in mice through immunohistochemistry. (D) The relative protein levels of EMT-associated proteins in mice. € Transmission electron microscopy of renal tubular epithelial cells in *db/m* mice. (F) The relative protein levels of laminin and type IV collagen in HK-2 cells cultured with NG by immunofluorescence. (G)The relative protein levels of EMT-associated proteins in HK-2 cells. *db/m* + NS: *db/m* mice were injected with 0.9% normal saline via the tail vein; *db/m* + LV-NC: *db/m* mice were injected with vehicle lentivirus via the tail vein; and *db/m* + LV-YY1: *db/m* mice were injected with YY1-overexpression lentivirus via the tail vein. NG: cells treated with normal glucose 5.56 mmol/L; NG + Vehicle: cells treated with NG and transfected with vehicle lentivirus; and NG + YY1: cells treated with NG and transfected with YY1 overexpression lentivirus. Each bar represents the mean ± SD for animal groups of six and cell groups of three. $^{*}P < 0.05$ and $^{**}P < 0.01$, mean *db/m* + LV-YY1 vs *db/m* + NS or NG + YY1 vs NG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

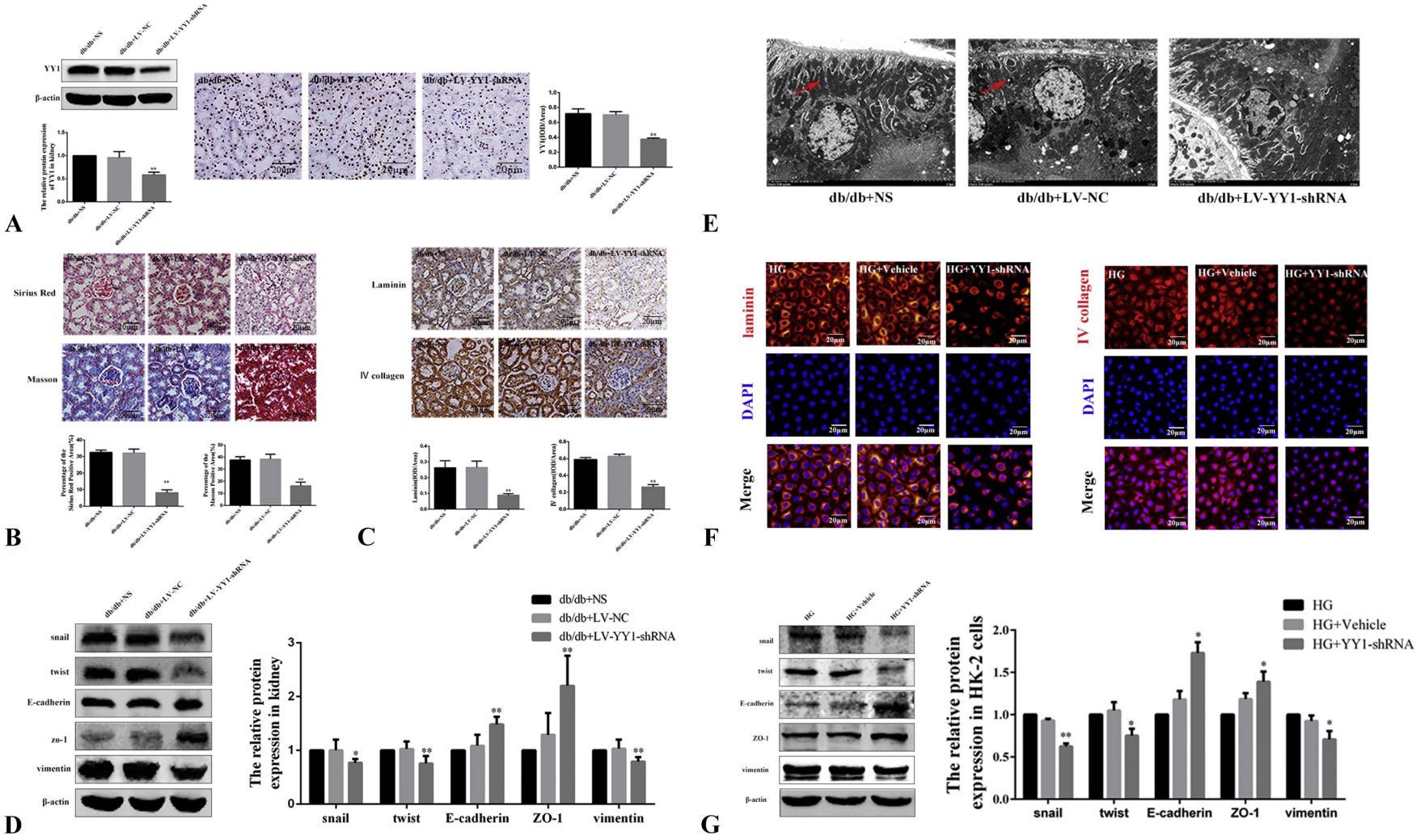

Fig. 7. Effects of YY1 knockdown on renal fibrosis in *db/db* mice and EMT of HK-2 cells. (A) The relative protein levels of YY1 in mice. (B) Masson staining and Sirius red staining of renal cortex sections of *db/db* mice. (C) The expression of laminin and type IV collagen in mice through immunohistochemistry. (D) The relative protein levels of EMT-associated proteins in mice. (E) Transmission electron microscopy of renal tubular epithelial cells in *db/db* mice. (F) The relative protein levels of laminin and type IV collagen in HK-2 cells cultured with HG by immunofluorescence. (G)The relative protein levels of EMT-associated proteins in HK-2 cells. *db/db* + NS: *db/db* mice were injected with 0.9% normal saline via the tail vein; *db/db* + LV-NC: *db/db* mice were injected with vehicle lentivirus via the tail vein; and *db/db* + LV-YY1: *db/db* mice were injected with YY1-shRNA lentivirus via the tail vein. HG: cells treated with HG 60 mmol/L. HG + Vehicle: cells treated with HG and transfected with vehicle lentivirus; and HG + YY1-shRNA: cells treated with HG and transfected with YY1-shRNA lentivirus. Each bar represents the mean ± SD for animal groups of six and cell groups of three. $^{*}P < 0.05$ and $^{**}P < 0.01$, mean *db/db* + LV-YY1-shRNA vs *db/db* + NS or HG + YY1-shRNA vs HG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Thus, our first goal was to confirm the relationships between HG and YY1 levels. The time- and dose-dependent increases in total YY1 expression and nuclear translocation confirmed that HG is a key inducer of YY1 in HK-2 cells. Initially, we chose 0 mmol/L, 30 mmol/L, 60 mmol/L and 90 mmol/L D-glucose to detect the expression changes of the total and nuclear YY1 protein in HK-2 cells. However, we finally replaced 0 mmol/L with 5.56 mmol/L as the lowest concentration gradient owing to the glucose needs of HK-2 cells for growth (Supplemental Fig. 1). Our research group has confirmed that HG is a key inducer of EMT and apoptosis in HK-2 cells and/or NRK-52E cells by using 30 or 60 mmol/L D-glucose [11,44]. Considering the toxicity of high concentration of glucose, we finally chose 60 mmol/L in the following research after confirming that HG was a key inducer of YY1 in HK-2 cells. In order further verify all the observed changes were the results of HG itself, and not of osmotic pressure changes induced by HG, an osmotic pressure control was included in our studies; the results ruled out the possibility of changes in osmotic pressure in vitro (Supplemental Fig. 2).

Study Our previous research showed that Akt/mTOR signaling participates in the ethanolic *Ginkgo biloba* leaf extract-mediated prevention of renal fibrosis in DN [12]. In addition, our other studies also found that mTOR/P70S6K signaling is activated in the brain and renal cortex of diabetic rats, and the latter further promotes the EMT of DN [11,43]. Research has shown that the phosphorylation of tubulin/p70S6K is enhanced by HG, leading to apoptosis of tubular epithelial cells in the kidney cortex of rats, mediated in part by cleavage of poly (ADP-ribose) polymerase (PARP) by YY1 [38]. Thus, we tested whether the overexpression and altered nuclear translocation of YY1 were induced by HG-mediated activation of the mTORC1/P70S6K signaling pathway. Following treatment with rapamycin, the expression of YY1 and redistribution of YY1 from the nucleus to the cytoplasm was significantly decreased, confirming our speculation of the role of YY1 in HG-cultured HK-2 cells.

Further molecular mechanism studies were carried out to test the mechanism by which YY1 may regulate renal fibrosis in DN. As a mesenchymal protein, α-SMA is a hallmark of myofibroblasts and is well-regarded as a biomarker of renal fibrosis. Although YY1 can bind to the α-SMA promoter and promote the formation of neonatal pulmonary hypertension and lung fibroblasts, the impact on α-SMA promoter activity and the corresponding precise mechanism remain unknown [41,44,45]. The increased expression of α-SMA stimulated by HG and overexpression of YY1, together with the reduction in α-SMA levels induced by small interfering RNA, supported the hypotheses that YY1 can directly upregulate α-SMA in HG-cultured HK-2 cells. To further verify these phenomena in vivo, mice were treated with lentivirus via tail vein injection with the purpose of knock-downing or overexpressing YY1. Green fluorescence in the kidney showed that the lentivirus invaded the kidney, indicating the success of gene editing in the mouse model (Supplemental Fig. 3). Based on the results from the mouse models, the consistent changes in α-SMA expression stimulated by YY1 overexpression and small interfering RNA treatment in mice confirmed the close relationship between YY1 and α-SMA (Supplemental Figs. 4, 5). Subsequently, in-depth ChIP assays and luciferase reporter assays were conducted, and the final results showed that YY1 directly regulated α-SMA expression by binding to the α-SMA promoter.

After identifying YY1 as a key player in the renal fibrosis process of DN, we further determined the regulatory mechanism of fibrosis mediated by YY1. It is well known that EMT promotes advanced renal fibrosis, which is characterized by glomerulosclerosis and renal interstitial fibrosis [46–48]. The loss of epithelial markers such as E-cadherin and gain of mesenchymal markers such as vimentin indicate that cells are undergoing EMT, which further regulates target genes, such as snail family members, resulting in the increased expression of collagens and fibronectin [49–51]. Reports have established that YY1 is involved in the regulation of EMT in cancers, along with the regulation of snail [30,52,53]. In this study, we found that HG promoted EMT-associated protein expression in a time- and dose-dependent manner (Supplemental Fig. 6). Our results also showed that YY1 overexpression increased the levels of Cr, BUN and urinary albumin, Masson and Sirius red staining, expression of laminin and type IV collagen and promoted renal fibrosis in DN in mice. Therefore, the changed expression of ZO-1, E-cadherin, vimentin, α-SMA, snail and twist in YY1 lentivirus-treated mice and HK-2 cells showed that YY1 promoted renal fibrosis in the initiation of EMT in DN. Notably, renal fibrosis in *db*/*db* mice was also reversed by treatment with rapamycin, which further inhibited HG-induced EMT in vivo and in vitro (Supplemental Fig. 7).

Considering the direct effects of YY1 in DN fibroblasts, decreasing YY1 expression may inhibit renal fibrosis and help to protect against diabetic nephropathy-induced renal fibrosis by the mechanism of EMT. In support of this idea, we investigated the effect of knocking down YY1 in *db*/*db* mice by injection of a YY1-targeting shRNA lentivirus via the tail. Compared to that of *db*/*db* mice, the improved kidney function and renal fibrosis in YY1-knockdown mice indicated that knocking down YY1 could effectively improve renal fibrosis in DN. After confirming the improvement of EMT induced by downregulating YY1 in vivo, further investigations were performed in HK-2 cells to uncover the role of YY1 in the EMT process of DN; the restoration of the expression of EMT-associated proteins in the HG + YY1-shRNA group proved our previous hypotheses. Therefore, our data suggest that YY1 could be an emerging treatment target for renal fibrosis of DN.

Accumulating studies have indicated that mitochondrial dysfunction is central to the pathogenesis of DN and that mitochondria might be a novel therapeutic target in DN [54–56]. In addition, mitochondrial oxidative function can also be controlled by mTOR via the YY1-PGC-1α transcriptional complex, and knockdown of YY1 causes a significant decrease in mitochondrial gene expression and respiration [57]. According to the results of transmission electron microscopy in our study, the abnormalities of the subcellular structure of renal tubular epithelial cells, especially mitochondrial dysfunction, might be the result of an injury to the renal tubular cell organelles induced by the upregulated YY1 expression in DN. In addition, a recent study reported that mitochondrial dysfunction is a novel potential driver of EMT in cancer [58]. Thus, whether mitochondrial dysfunction is also involved in the HG-induced EMT process of DN via mTORC1/P70S6K-dependent YY1 overexpression has not been fully determined, and is the subject of ongoing investigation.

Clinical studies of YY1 in nephropathy or fibrosis have been limited by strict clinical trials and ethical oversight. Previous studies have shown that YY1 protein expression is upregulated in the lungs of

Table 1
Effects of YY1 on the body weight, kidney index, FBG, Cr, BUN and mAlb levels of *db*/*db* mice. *db*/*db* + NS: *db*/*db* mice were treated with 0.9% normal saline; *db*/*db* + LV-NC: *db*/*db* mice were treated with control lentivirus (LV-NC); *db*/*db* + LV-YY1-shRNA: *db*/*db* mice were treated with YY1 shRNA lentivirus (LV-YY1).

Group	BW	Kindey index	FBG	Cr	BUN	mAlb/Cr
	(g)	(mg/kg)	(mmol/L)	(μmol/L)	(mmol/L)	(mg/mmol)
db/*db* + NS	52.33 ± 2.19	7.87 ± 0.55	15.90 ± 6.72	52.38 ± 8.99	8.47 ± 0.50	14.53 ± 0.91
db/*db* + LV-NC	54.12 ± 3.85	8.00 ± 0.45	15.55 ± 4.07	51.31 ± 7.82	8.85 ± 0.24	14.90 ± 0.92
db/*db* + LV-YY1-shRNA	44.73 ± 5.82**	9.47 ± 1.02**	11.12 ± 2.25	37.64 ± 8.08*	7.66 ± 0.92*	10.88 ± 1.41**

Data are presented as the mean ± SD.
*$P < 0.05$, **$P < 0.01$, means *db*/*db* + LV-YY1-shRNA vs *db*/*db* + NS.

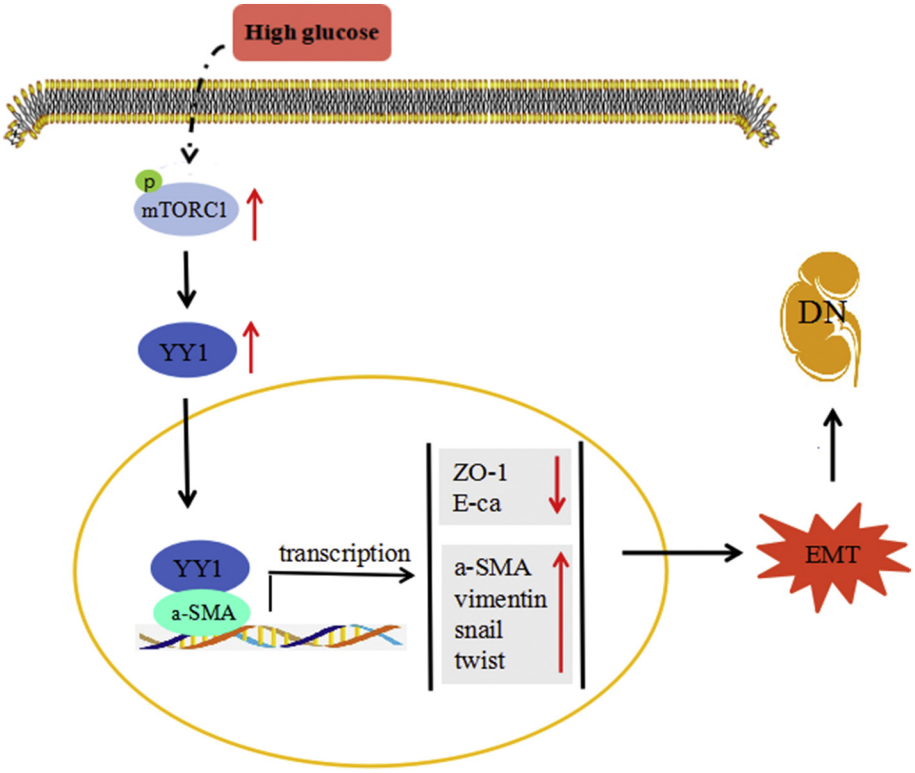

Fig. 8. Proposed model of HG-induced renal fibrosis in diabetic nephropathy. Possible mechanisms of mTORC1/p70S6K-mediated YY1 expression-induced diabetic nephropathy by upregulation of α-SMA and of the process of EMT.

patients with idiopathic pulmonary fibrosis (IPF) [32]. Another clinical YY1 experiment was carried out in a small cohort of kidney transplant recipients to investigate the organ protective mechanism of chronic kidney failure [59]. Our studies are consistent with the abovementioned results; thus, YY1 could be an emerging therapeutic strategy for patients with DN. Although HK-2 cells were chosen for the mechanistic study of YY1 in DN in our studies, the pharmacodynamic evaluation was based on rodent models due to the almost impossible operability of DN clinical patients in mainland China. Thus, in-depth clinical studies are needed to further strengthen the evidence that targeting YY1 may be a promising therapeutic approach for renal fibrosis of diabetic nephropathy.

5. Conclusion

In summary, our results demonstrated that mTORC1/P70S6K-dependent YY1 overexpression and nuclear translocation were responsible for HG-induced up-regulation of α-SMA and subsequently promoted renal fibrosis via dysregulation of EMT-associated proteins (Fig. 8). The inhibition of mTOR or knockdown of YY1 markedly reduced HG-induced renal fibrosis in diabetic nephropathy. Overall, the current study provides novel insight into the pathogenesis of HG-induced mitigation of renal fibrosis in diabetic nephropathy. Thus, targeting YY1 may be a promising therapeutic strategy for renal fibrosis of diabetic nephropathy and other metabolic-related fibrosis diseases.

Author's contributions

The experiments of this work were designed by Dr. Yin, Dr. Lu and Tingting Yang. The experiments were performed by Fanglin Shu, Hao Yang, Cai Heng and Yi zhou. Tingting Yang and Fanglin Shu analyzed the data and wrote the manuscript. Tingting Yang, Fanglin Shu and Hao Yang edited of the figures and Tables. Tingting Yang, Dr. Yin and Dr. Lu reviewed the manuscript. Rest of the authors either supplied reagent or helped in treatments to mice and cells.

Conflict of interest

The authors declared that no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2019.04.013.

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