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Picroside II alleviates renal fibrosis through YY1-dependent transcriptional inhibition of TGFβ1

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ABSTRACT

Diabetic Nephropathy (DN) has become the leading cause of end-stage renal disease worldwide. Studies have indicated that Transforming Growth Factor beta1 (TGFβ1) is the most potent factor contributing to renal fibrosis, and understanding the exact pathogenic mechanism of renal fibrosis is crucial for alleviating the condition. Previous research has identified Yin Yang 1 (YY1) as an effective inhibitor of TGF-β1. Our study, through dual-luciferase reporter gene assays and Western blot experiments, screened and obtained the small molecule compound PdII. Subsequently, validation in a high-glucose-induced renal mesangial cell injury model showed that PdII treatment significantly increased the expression of YY1 protein and mRNA, while correspondingly reducing the expression of TGFβ1 protein and mRNA. Dual-luciferase reporter gene assay results revealed that, compared to the control group, the luciferase transcription activity of YY1 molecules increased in the PdII treatment group, and the luciferase transcription activity of TGFβ1 decreased. By further designing mutations in the binding sites between TGFβ1 and YY1 on the promoter, transfecting fluorescent enzyme reporter gene plasmids with TGFβ1 mutant promoter into mesangial cells damaged by high glucose, and then treating the cells with PdII, it was observed that the luciferase transcription activity of TGFβ1 did not decrease. Therefore, these results suggest that PdII may inhibit TGFβ1 transcriptional activity by activating YY1, thereby slowing down the progression of diabetic nephropathy.

1. Introduction

Hyperglycemia could cause nephropathy, neuropathy, retinopathy and macrovascular complications [1]. Diabetic Nephropathy (DN) has become the leading cause of end-stage renal disease worldwide [2]. Despite a similar duration of diabetes and similar blood glucose control levels, about 20–40 % of diabetic patients are at high risk for nephropathy, while the remaining diabetic patients are less prone to develop DN [3], and differences in the expression of protective endogenous factors might contribute to differential susceptibility to DN in the two populations.

Hyperglycemia can lead to glomerular abnormalities in DN patients, with excessive secretion of transforming growth factor-β 1 (transforming growth factor-β 1, TGFβ1) causing the most severe damage to mesangial cells [4]. Previous studies have established that TGFβ1 is the main causative factor of DN [5]. Excess TGFβ1 promotes the accumulation of extracellular matrix proteins, such as fibronectin and collagen IV, which leads to glomerular fibrosis [6]. Podocyte apoptosis caused by TGFβ1 further increased the permeability of the glomerular basement membrane and aggravated diabetic proteinuria and renal fibrosis [7]. These pathological changes promoted by TGFβ1 aggravated the progression of DN. Therefore, inhibition of TGFβ1 is considered a promising therapeutic target for the treatment of DN. Despite substantial efforts have been devoted in therapeutic approaches targeting TGFβ1, clinical trials

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have shown that none of these methods provide satisfactory results [8].

Previous studies have shown that some transcription factors involved in the pathological mechanism of DN can be overactivated by hyperglycemia or advanced glycation end products [9–11]. Due to the importance of transcription factors in DN progression and TGF-β1 regulators might have critical renoprotective functions in individuals with mild DN, identifying endogenous transcriptional repressors targeting TGFβ1 would be of great benefit for the treatment of diabetic nephropathy.

Previously, we have demonstrated that Yin Yang 1 (YY1) is an effective repressor of TGFβ1 [13]. YY1 directly binds to the TGFβ1 promoter region and inhibits TGFβ1 transcription [12] in human renal mesangial cells. In the mouse model, YY1 increased in glomerular mesangial cells in the early stage of diabetic nephropathy and decreased in the later stage; knockout of YY1 in the kidney exacerbates glomerular sclerosis, while overexpression of YY1 attenuates glomerulosclerosis [12]. Furthermore, patients with high YY1 expression developed diabetic nephropathy [12] than those with low YY1 expression. These results suggest that YY1, may be a potent transcriptional repressor of TGFβ1 during DN development.

Neopicrorhiza scrophulariiflora (Pennell) D. Y. Hong is a traditional Chinese herbal medicine in China. Its main bioactive ingredient is a glycoside derivative, called picroside II (Pd II) [13]. In the past decades, numerous studies have shown that Pd II has antioxidant, anti-inflammatory and anti-apoptotic activities, and has some therapeutic potential in the prevention and treatment of organic ischemia/reperfusion injury, kidney injury, liver injury, inflammation, tumor metastasis, angiogenesis, etc. [13]. More studies have confirmed that Pd II can reduce renal fibrosis [14]. Therefore, we speculated that Pd II might contribute to the improvement of diabetic nephropathy. The aim of the present study was to explore that Pd II might reduce TGFβ1 levels and ultimately reduce renal fibrosis through promoting the expression of YY1.

2. Materials and methods

2.1. Establishment of Diabetic Renal Injury Cell Model and Drug Administration

Transfer HRMC cells into three 12-well plates, with 500 μL of culture medium per well. Once the cell growth density reaches 30 %, subject them to starvation treatment for 6 h. Then divide the cells into three groups, with three replicate wells for each group, and label the groups on the lid of the 12-well plate. For the low glucose control group, switch the cells to low glucose medium; for the high glucose control group, switch to high glucose medium; for the high glucose plus drug group, initially add only the high glucose medium and treat for 24 h. To the low glucose control group, add PBS (equal in volume to PdII), and similarly add PBS to the high glucose control group. For the high glucose plus drug group, add PdII to achieve a final concentration of 30 μM and treat for 24 h. Afterwards, remove the cell culture plate, aspirate the medium, quickly plunge into liquid nitrogen for instant freezing, and then store at −80 °C for later protein and RNA extraction.

2.2. Western blotting

The total protein from the cells was extracted using ice-cold RIPA lysis buffer (Beyotime, Shanghai, China) supplemented with a protease inhibitor cocktail and a phosphatase inhibitor cocktail (both from Sigma-Aldrich, USA). Equivalent amounts of protein were separated on 10 % or 12 % SDS-PAGE, transblotted onto 0.22-mm PVDF membrane (Merckmillipore, Burlington, MA, USA). Membranes were blocked with 5 % BSA (sigma, USA) in TBST buffer for 1 h or 2h and incubated with the indicated primary antibodies with gentle rocking overnight at 4 C. Membranes were further incubated with the corresponding HRP-conjugated secondary antibodies (BioTNT, 1:5000) for 1 h at room temperature. In the final step, the protein bands were visualized using enhanced chemiluminescence detection kit (Pierce ECL Plus, Thermo Scientific) and the resulting images were meticulously analyzed a luminescent image analyzer (Image Quant LAS 4000 or Amersham Imager 600, GE Healthcare Life Sciences).

2.3. RNA isolation and RT-qPCR

Total RNA was isolated from frozen tissues or cultured cells using Trizol reagent (Ambion, USA), subsequently reverse-transcribed, and analyzed by RT-qPCR using commercial kits (Vazyme Biotech, Nanjing, China). The experiments were carried out using a 384-well format on a LightCycler 480 II Instrument (Roche Diagnostics, Mannheim, Germany). The relative abundance of mRNA was assessed and normalized against ribosomal 18S RNA or cyclophilin A through the 2-DDCt method. The specific primer sequences utilized for these reactions are detailed in Table 1.

2.4. Immunofluorescence

The circular cell smear was soaked in alcohol for 6 h, rinsed twice with PBS, and then placed in a 12-well plate. HRMC cells were putted into the 12-well plate. The cells were treated according to the "Establishment of Diabetic Renal Injury Cell Model and Drug Administration". After rinsing the treated cell smear with warm PBS, 500 μL of precooled acetone was added to each well and left to fix on ice for 10 min. Discard the acetone and rinse twice with PBS, 5 min each time. Remove the PBS and add 500 μL of 1 % BSA to each well, and incubate in a 37 °C incubator for 20 min. Remove the cell smear and place it on a glass slide, ensuring that the correct side is facing up. Carefully add primary antibody, anti-YY1(1:200, Proteintech), and incubate overnight at 4 °C. The next day, remove the cell smear and wash with PBS three times. Add the secondary antibody, avoid light exposure, and incubate at 37 °C for 1 h. Remove the cell smear and wash with PBS three times. Restain with DAPI: dilute DAPI in PBS at a 1:1000 ratio and stain for 10 min. Wash with PBS three times. Prepare the glass slide and seal with transparent nail polish. Observe under a fluorescence microscope (Carl Zeiss AG).

2.5. Site-directed mutagenesis and dual luciferase reporter gene assay

Genomic DNA was extracted and used as a template for PCR to clone the target segments. Fragments containing the TGFβ1 promoter or the YY1 promoter were inserted into the pGL4.22 vector with the firefly luciferase reporter gene. This resulted in the construction of reporter gene plasmids, YY1-luciferase, and TGFβ1-luciferase. These plasmids were separately transfected into human renal mesangial cells (HRMC) using Lipofectamine 3000 transfection reagent (Invitrogen

Table 1
The primer sequences used in the experiment.

Name	Species	Primer sequence
18S	Human	Forward: TTCGAACGTCTGCCCTATCAA Reverse: ATGGTAGGCACGGCGACTA
FN	Human	Forward: TGCACATTGCCTGTTCTGCT Reverse: TGCATCTTGGTTGGCTGCAT
TGFβ1	Human	Forward: TGCACATTGCCTGTTCTGCT Reverse: TGCATCTTGGTTGGCTGCAT
YY1	human	Forward: AAGAGCGGCAAGAAGAGTTAC Reverse: CAACCACTGTCTCATGGTCAATA
18s	Mouse	Forward: CGCCGCTAGAGGTGAAATTCT Reverse: CATTCTTGGCAAATGCTTTCG
Yy1	Mouse	Forward: AAGAGCGGCAAGAAGAGTTAC Reverse: CAACCACTGTCTCATGGTCAATA
Tgfβ1	Mouse	Forward: TAAAGAGGTCACCCGCGTGCTAAT Reverse: ACTGCTTCCCGAATGTCTGACGTA
Fn	Mouse	Forward: ACGGTTTCCCATTACGCCAT Reverse: CTAGGTAGGTCCGTTCCCACT

Corporation). After 24 h of transfection, cells were treated with the compound Pd II (30 μM) for an additional 24 h. Fluorescence intensity of YY1-luciferase and TGFβ1-luciferase was measured using the Dual-Luciferase® Reporter Assay System (Promega Corporation). This was done to validate the mechanisms by which the compound regulates YY1 and TGFβ1 at the transcriptional level.

2.6. Chromatin immunoprecipitation (ChIP) and sequential ChIP assay

Briefly, HRMC cells with treatments were lysed in lysis buffer and sonicated (15s on and 90 s off, repeated 8 times). After precipitation with Agarose A for 30 min, the fragmented DNA was pulled down with YY1 antibodies and then subjected to amplification by qPCR. Afterwards, sequential ChIP assays were performed (Millipore, USA). The specific primer sequences utilized for these reactions are detailed in Table 2, which referring to a previous investigation by Gao P et al. [12].

3. Result

3.1. Pd II promoted YY1 expression while reducing TGFβ1 expression in renal mesangial cells

To validate the in vitro effects of Pd II, a high-glucose-induced HRMCs cell model was constructed. After treating with PdII for 24 h, changes in the expression of YY1, TGFβ1, and FN proteins and mRNAs were detected using Western blot (WB) and quantitative real-time polymerase chain reaction (qRT-PCR). Fig. 1A shows the compound structure of Pd II. In Fig. 1B–E, the results of the Western Blot and protein quantification demonstrate that compared to the control group with normal cell culture conditions, the high glucose stimulation in the model group leads to an upregulation in the protein expression levels of FN and TGFβ1 ($^*P < 0.05$). However, following treatment with Pd II, there is a downregulation in the expression of the fibrotic factor TGFβ1 and its downstream protein FN ($\#P < 0.05$) compared to the model group. Additionally, the expression of YY1 protein is upregulated ($\#P < 0.05$). Fig. 1F–I shows the qRT-PCR results. Compared to the control group, the mRNA expression of YY1, TGFβ1, and FN in the model group is significantly upregulated ($^*P < 0.05$). In comparison to the model group, the experimental group demonstrates a downregulation in the expression of TGFβ1 and FN and an upregulation in YY1 expression ($\#P < 0.05$).

3.2. Pd II can improve diabetic nephropathy (DN) at the transcriptional level

To investigate the mechanisms underlying the transcriptional regulation of YY1 and TGFβ1 by Pd II, we constructed reporter gene plasmids YY1-luciferase and TGFβ1-luciferase and employed a reagent kit to measure the fluorescence intensity of YY1-luciferase and TGFβ1-luciferase. As shown in Fig. 2A, high glucose stimulated the transcriptional activity of the TGFβ1 gene. Conversely, the introduction of PdII resulted in a decreased transcriptional activity of the TGFβ1 gene under high glucose conditions, indicating a protective effect of Pd II on mesangial cell fibrosis in the context of high glucose-induced renal injury. Fig. 2B illustrates the results of HRMC cells transfected with YY1-luciferase. As depicted, under high glucose stimulation without the addition of the compound, the transcriptional activity of the YY1 gene increased. However, upon the addition of the compound, the transcriptional activity of the YY1 gene further increased, suggesting that the protective effect of Pd II on the fibrosis of glomerular mesangial cells in DN patients may be achieved by elevating the transcriptional level of YY1.

Table 2
The qPCR primers for the TGFβ1 promoter region [12].

Target gene	Primer sequence
TGFβ1 wt	Forward: 5′-AGAACAGAGTCTAGCACAGG-3′ Reverse: 5′-TCCCAGGTAACCATCATG-3′
TGFβ1 YY1-mut	Forward: 5′-ACTCAGGGTGAAAGCCGTAGTCTTAGCATG-3′ Reverse: 5′-GTAGTCTTAGCATGCATCACAAGG-3′

3.3. Pd II exerts its protective effects on diabetic nephropathy (DN) by promoting the binding of YY1 to TGFβ1

After mutating the binding sites on the YY1 and TGFβ1 promoters, a mutated promoter for TGFβ1 was designed, and this mutated TGFβ1 promoter was inserted into a reporter gene plasmid vector. Cells were transfected with this construct. In Fig. 3A, it can be observed that, after mutating the binding sites, high glucose stimulation significantly increased the transcription level of TGFβ1 ($^*P < 0.05$). However, when cells were treated with Pd II under these conditions, there was no significant downregulation of TGFβ1 transcription (ns). This suggests that after mutating the binding sites, Hesperetin II no longer inhibits TGFβ1 transcription. Fig. 3B provides an illustration of the mutated binding site. In Fig. 3C, the "yy1" represents the experimental group. The bar graph shows PCR results. When compared to the control group, it is evident in the high glucose group that YY1 and TGFβ1 interact ($^*P < 0.05$). In the experimental group treated with Pd II, the interaction between YY1 and TGFβ1 is strengthened ($\#P < 0.05$). This suggests that YY1 protein can pull down more TGFβ1 through their interaction. The lower part of the bar graph corresponds to the DNA gel where the PCR products were run. Fig. 3D shows the model illustrates that YY1 functions by binding to the TGFβ1 promoter.

3.4. Pd II treatment of human kidney mesangial cells cultured in high glucose promotes YY1 gene expression in the nucleus

After revealing that Pd II could improve the fibrosis of kidney mesangial cells, increase YY1 and reduce the expression level of TGFβ1, it was found in further study that it could promote YY1 gene expression in the nucleus of human kidney mesangial cells when cultured with high glucose combined with Pd II. As shown in Fig. 4A, DAPI serves as a marker for cell nuclei, YY1 represents the staining of YY1 molecules, and Merge combines DAPI and YY1 images to distinguish whether the two fluorescent signals overlap at the same cellular location. It can be observed that, compared to the control group, cells treated with the compound show an increase in fluorescent signals of YY1 in the cell nucleus. This indicates that Pd II leads to enhanced YY1 expression in the cell nucleus, and this process primarily occurs within the cell nucleus. In Fig. 4B, YY1's subcellular localization is further validated. The results of protein quantification are shown in Fig. 4C ($^*P < 0.05$). The immunoblotting results demonstrated that YY1 is primarily localized in the cell nucleus, and Pd II enhances the nuclear YY1 expression.

4. Discussion

Approximately 30%–40 % of diabetic patients eventually develop nephropathy. Diabetic nephropathy (DN) has become a major cause of end-stage renal disease. However, the exact pathogenesis of type 2 diabetic nephropathy remains unclear. Previous research has primarily focused on factors such as disordered glucose and lipid metabolism, hemodynamic factors, oxidative stress, and genetic factors. In recent years, both animal experiments and clinical studies increasingly suggest that inflammation plays a crucial role in the occurrence and continuous progression of diabetic nephropathy. Studies have demonstrated that TGF-β1 plays a key regulatory role in the development of diabetic nephropathy. As a potent fibrogenic factor, TGF-β1 can induce the expression and deposition of extracellular matrix components, such as fibronectin and Type IV collagen (COL4), in the glomerulus. It also inhibits the expression of extracellular matrix metabolic enzymes, stimulates the production of metalloproteinase inhibitors, leading to increased extracellular matrix synthesis, decreased degradation, and

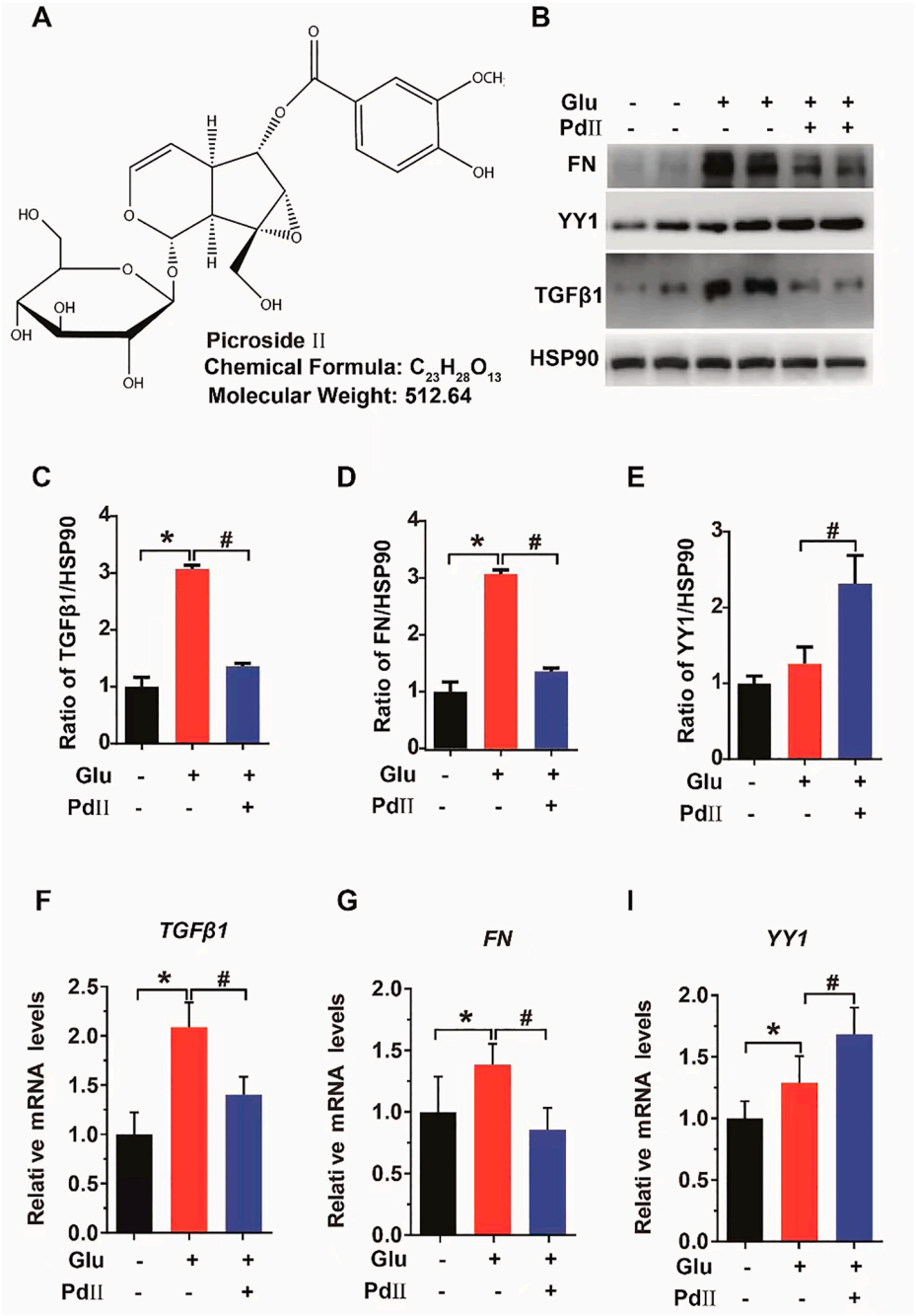

Fig. 1. Pd II reduces FN and TGFβ1 expression and promotes YY1 expressing in HRMCs. HRMC cells were pretreated glucose (30 mM) and followed by w/o Pd II (30 μM) treatment for 24 or 48 h. (A) The structure of Pd II. (B)Western blot analyses revealing Pd II could downregulate FN and TGFβ1 expression in high glucose circumstances. (C–E) Graphs showing the quantitative analysis for FN and TGFβ1, YY1 protein expression using image J and normalized to HPS90. (n = 3); (F–I) Real time PCR analysis for FN and TGFβ1, YY1 mRNA expression in the kidney tissue among groups (n = 4). *P < 0.05 compared to with low glucose group (5.5 Mm), #P < 0.05 compare with cells treated with high glucose group (30 mM).

ultimately resulting in glomerulosclerosis and interstitial fibrosis [15].

In our preliminary screening of a library of traditional Chinese medicine monomers, we found that picroside II (PdII) can reduce the elevated expression of TGF-β1 in renal mesangial cells under high glucose stimulation. This result has been validated at both the protein and mRNA levels. In terms of liver function, studies by Li Shuang et al. have shown that PdII can improve liver function, inhibit lipid peroxidation reactions, downregulate the expression of TGF-β1 and Type I collagen mRNA and protein, thereby inhibiting the formation of liver fibrosis. In the kidney, other research suggests that in a rat model of renal ischemia-reperfusion, the degree of inflammation and tissue fibrosis in renal tissues is reduced after treatment with PdII, indicating that PdII has an anti-fibrotic effect on renal ischemia in rats, possibly achieved through the inhibition of renal inflammation [16]. Additionally, PdII has a strong anti-apoptotic effect on acute kidney injury (renal ischemia-reperfusion model) [17]. However, in the field of diabetic nephropathy, the role of PdII has not been reported.

In the HFD/STZ-induced diabetic nephropathy mouse model, Western blot analysis revealed that, compared to the control group, the PdII treatment group exhibited increased expression of YY1, decreased expression of TGF-β1 and fibronectin (FN). qRT-PCR results were consistent with the Western blot findings. Furthermore, previous

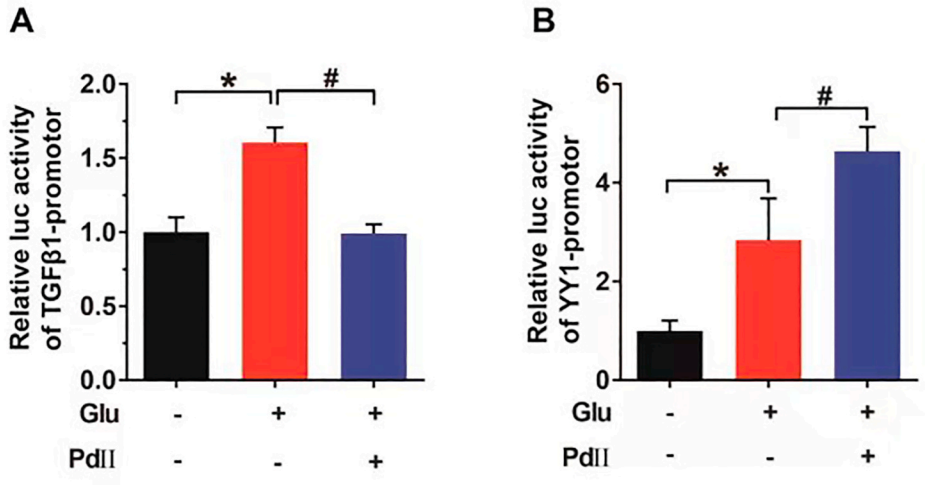

Fig. 2. Effect of Pd II on YY1 expression and distribution in HG-treated HRMCs by immunofluorescence and Western blot among different groups as indicated. (n = 4). (A) HRMC were stained with blue DAPI to visualize the cell nuclei. (B)Western blot analyses showing the abundance of YY1 in HG-treated HRMCs after 24 h treatment of Pd II. The gels were run under the same experimental conditions. (n = 4). Lamin A/C is the internal reference protein of nucleus. β-Tubulin is the internal reference protein of cytoplasm.

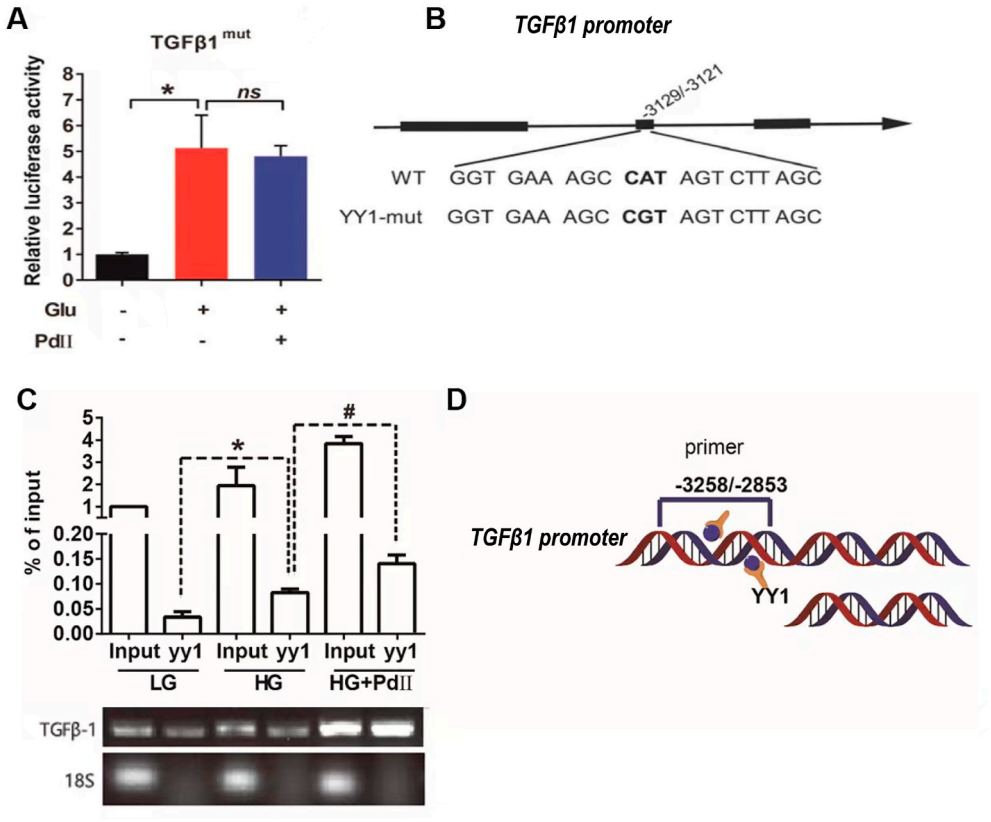

Fig. 3. Pd II cannot regulated TGFβ1 transcription after mutation of YY1-binding site with TGFβ1 and negatively regulates TGFβ1 transcription through direct bonding to the TGFβ1 promoter. (A) Luciferase reporter assays of mutated TGFβ1 (TGFβ1 YY1-mut) among groups. mut, mutant. ns, no significance. (B) The scheme of point mutation construction in the TGFβ1 promoter (TGFβ1 YY1-mut). *P < 0.05 compared to with low glucose group (5.5 mM). (C) ChIP-PCR analysis of TGFβ1 promoter by YY1 antibody. (D) A model shows that YY1 worked through binding to the TGFβ1 promoter. Significance was determined by Student's *t*-test. *P < 0.05 compared to with low glucose group (5.5 mM), #P < 0.05 compare with cells treated with high glucose group (30 mM). n = 3.

research has found that the key nuclear transcription factor YY1 can synergistically inhibit the expression of TGF-β1. In tissues from diabetic nephropathy patients at different clinical stages, YY1 is highly expressed in the early stages of the disease, decreasing as glomerular fibrosis progresses in the later stages. We found that PdII can activate the expression of YY1 molecules.

In previous studies, YY1, a key nuclear transcription factor, has been identified to cooperatively inhibit the expression of TGFβ1. Across different clinical stages of diabetic nephropathy, YY1 is highly expressed in the early disease stages, decreasing as glomerular fibrosis progresses. Our study reveals that PdII activates YY1 expression. Initially investigating Pd II's regulatory effect on YY1 transcription levels, we observed

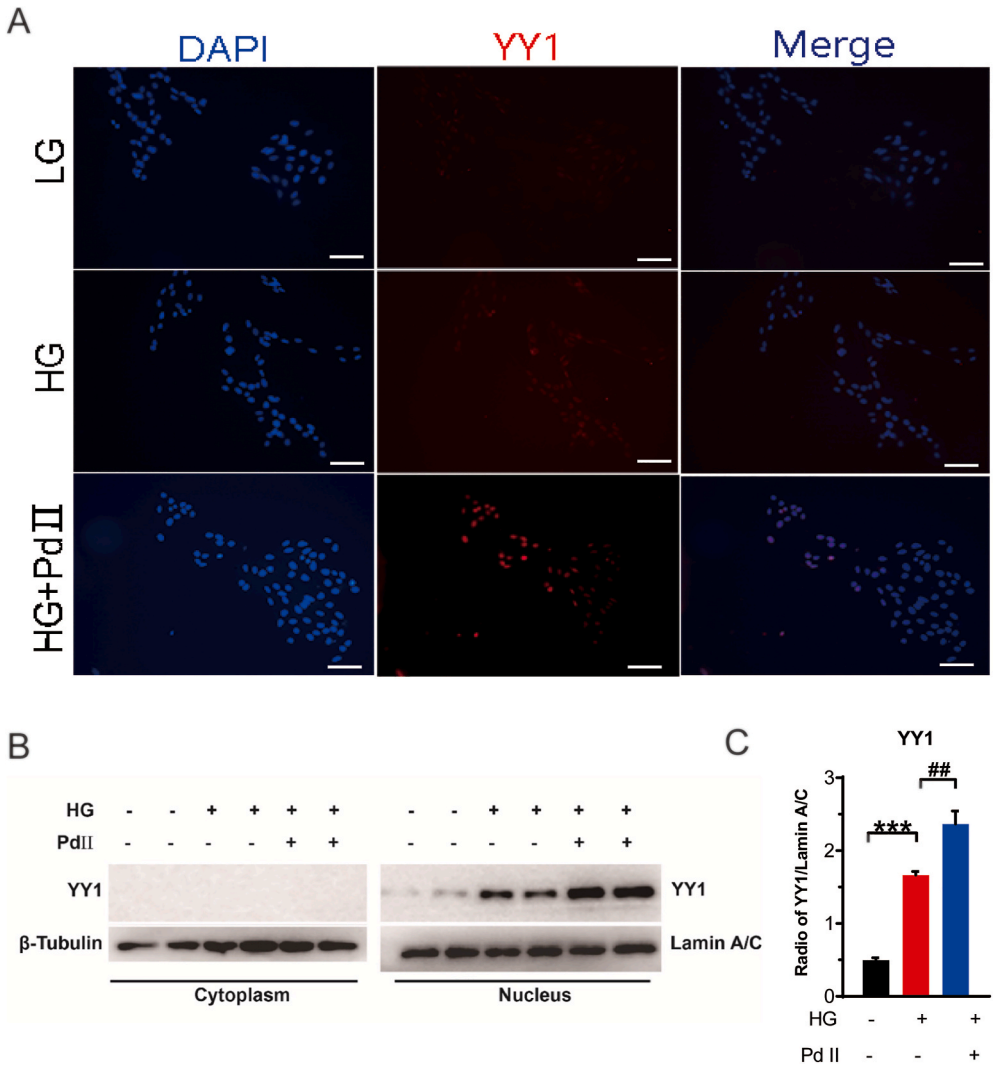

Fig. 4. Pd II regulates TGFβ1 transcription and YY1 transcription in HRMCs.
(A) HRMCs were transfected with TGFβ1-promoter luciferase plasmid and the luciferase reporter activity of TGFβ1 and protein levels after stimulated by Pd II (30 μM) were analyzed. (B) Luciferase reporter assays of YY1 among groups in HRMCs. Significance was determined by Student's *t*-test. *P < 0.05 compared to with low glucose group (5.5 mM), #P < 0.05 compare with cells treated with high glucose group (30 mM). (C) Graphs showing the quantitative analysis for YY1 protein expression using image J. (n = 3).

an increase in YY1 transcriptional activity. Additionally, Pd II inhibits the transcriptional activity of TGFβ1. Mutation of YY1's binding site on TGFβ1, in a dual-luciferase reporter gene assay, showed Pd II no longer inhibited TGFβ1 transcriptional activity. Under high glucose stimulation and drug treatment, TGFβ1 transcriptional activity remained high, suggesting Pd II's inhibition of TGFβ1 involves YY1. Chromatin immunoprecipitation and immunofluorescence techniques demonstrated elevated RNA and fluorescence signals of TGFβ1 in the Pd II-treated group. YY1 molecules were predominantly in the cell nucleus, indicating Pd II inhibits TGFβ1 by enhancing YY1 transcription in the nucleus, thereby improving kidney function.

Our study signifies, for the first time, that Pd II exhibits a protective effect against diabetic nephropathy (DN) by targeting YY1 to inhibit TGFβ1. This introduces a novel perspective on DN protection and paves the way for new applications of Pd II. Limitations of this study include the absence of validation at the animal level. Subsequent research could involve treating various diabetic nephropathy models in rodents, including genetic and diet-induced models, with Pd II to further confirm its therapeutic effects. Providing conclusive evidence at the tissue level for the signaling pathway and the amelioration of DN by Pd II is a crucial aspect of this study.

Disclosure statement

The authors report there are no competing interests to declare.

CRediT authorship contribution statement

Xianjing Zhang: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Jiarong Zhang:** Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Xiaojun Xu:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Data curation, Conceptualization. **Suzhen Chen:** Writing – review & editing, Validation, Investigation, Data curation. **Fei Gao:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

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