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Lentiviral Vector-Mediated FoxO1 Overexpression Inhibits Extracellular Matrix Protein Secretion Under High Glucose Conditions in Mesangial Cells

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ABSTRACT

Diabetic nephropathy is characterized by inordinate secretion of extracellular matrix (ECM) proteins from mesangial cells (MCs), which is tightly associated with excessive activation of TGF-β signaling. The forkhead transcription factor O1 (FoxO1) protects mesangial cells from hyperglycemia-induced oxidative stress, which may be involved in ameliorating the redundant secretion of ECM proteins under high glucose conditions. Here, we reported that high glucose elevated the level of p-Akt to attenuate endogenous FoxO1 bioactivities in MCs, accompanied with decreases in the mRNA expressions of catalase (CAT) and superoxide dismutase 2 (SOD2). Meanwhile, the expressions of major ECM proteins-FN and Col I-increased under high glucose conditions, in consistent with the activation of TGF-β/Smad signaling. By contrast, overexpression of nucleus-localized FoxO1 (insensitive to Akt phosphorylation) directly up-regulated the expressions of anti-oxidative enzymes, accompanied with inactivation of TGF-β/Smad3 pathway, as well as decreases of extracellular matrix proteins. Moreover, similar to those MCs overexpressed of nucleus-localized FoxO1 in high glucose conditions, MCs with down-regulation of FoxO1 by small interference-RNA under normal glucose conditions showed increased FN level and activated TGF-β/Smad3 pathway. Our findings link the anti-oxidative activity of FoxO1 and the TGF-β-induced secretion of ECM proteins, indicating the novel role of FoxO1 in protecting MCs under high glucose conditions. J. Cell. Biochem. 117: 74–83, 2016. © 2015 Wiley Periodicals, Inc.

KEY WORDS: EXTRACELLULAR MATRIX; FORKHEAD TRANSCRIPTION FACTOR O1; GLOMERULAR MESANGIAL CELL; LENTIVIRAL VECTORS; OXIDATIVE STRESS; TRANSFORMING GROWTH FACTOR-B PATHWAY

Diabetic nephropathy (DN), the most common microvascular complication of diabetes, is characterized by excessive proliferation of mesangial cells (MCs), deposition of extracellular matrix (ECM), and thickening of glomerular basement membrane (GBM) at an early stage of progression. The excessive accumulation of ECM proteins in the mesangium of glomerulus results in glomerulosclerosis and contributes to the genesis and development of DN [Mason and Wahab, 2003; Kolset et al., 2012].

Forkhead transcription factor O1 (FoxO1), a pivotal transcription factor belonging to Forkhead protein family, is involved in oxidative stress, cell cycle arrest, proliferation, and other biological processes [Behl et al., 2009; Rached et al., 2010; Kousteni, 2011; Liu et al., 2014]. Attenuation of FoxO1 bioactivity, especially loss of anti-oxidative activity, is associated with the progression of DN. Previous studies showed that FoxO1 bioactivity decreased in renal cortex tissues of streptozocin (STZ)-induced DN rats, accompanied by excessive deposition of collagen IV and fibronectin in the mesangium, whereas resveratrol ameliorated these pathological changes through FoxO1 activation [Wu et al., 2012]. Furthermore, FoxO inactivation has been implicated in MC dysfunction, which is tightly related to enhanced oxidative stress [Kato et al., 2006]. But the role of FoxO1 in ECM deposition under high glucose conditions remains unclear.

High glucose conditions induce a positive feed-back loop between transforming growth factor β (TGF-β)/Smad pathway and reactive oxygen species (ROS), leading to the up-regulation of ECM proteins which contributes to diabetic nephropathy, lung fibrosis,

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mitral valve remodeling, and other diseases [Li et al., 2003; Liu and Gaston, 2010; Sohn et al., 2012; Hagler et al., 2013]. Recent studies have indicated that FoxO1's role intersects with the TGF-β/Smad pathway. Several studies have demonstrated that activation of PI3K/Akt pathway results in the inactivation of FoxO1 bioactivity, as well as the enhancement of TGF-β/Smad-induced Col I expression in MCs. Conversely, treatment with the PI3K inhibitor LY294002 decreased Col I expression [Runyan et al., 2004; Kato et al., 2006; Medina et al., 2011]. Furthermore, expression of plasminogen activator inhibitor 1 (PAI-1), a pivotal regulator in restraining the transformation of plasminogen into plasmin, was markedly increased after TGF-β1 treatment, while direct over-expression of FoxO1 attenuated this effect [Jung et al., 2009]. Therefore, a link might exist between FoxO1, the TGF-β pathway, and ECM protein secretion. The objective of our study was to investigate the role and mechanism of FoxO1 in TGF-β/Smad pathway activation and ECM protein secretion in MCs under high glucose conditions.

MATERIALS AND METHODS

MATERIALS

Rat glomerular mesangial cells and 293T cells were stored in liquid nitrogen. pGLV4-GFP vector encoding nucleus-localized FoxO1 coding sequences (pGLV4-GFP/3A-FoxO1), siRNA-FoxO1 (pGLV4-GFP/siRNA-FoxO1), and pGLV4-GFP normal control plasmid were constructed by Genepharma Co., Ltd. (Shanghai, PRC); rabbit anti-FoxO1 from Abcam Co. (Cambridge, UK); rabbit anti-phospho-FoxO1 (Ser-256), rabbit anti-TGF-β, rabbit anti-TβRII, rabbit anti-Smad3 from Cell Signaling Technology (Danvers, MA); rabbit anti-Akt1, rabbit anti-phospho-Akt1(Ser-473), rabbit anti-TβRI (ALK-5), rabbit anti-phospho-Smad3 (Ser-425) from Biobasic Inc. (Ontario, Canada). Primers were designed and synthesized by Shanghai Sangon Biotech Co. (Shanghai, PRC). Rat fibronectin and collagen I ELISA kits were purchased from CUSABIO Biotech Co. (Wuhan, China). CM-H2DCFDA probe was purchased from Molecular Probes (Eugene, OR).

CONSTRUCTION OF NUCLEUS-LOCALIZED FOXO1 LENTIVIRAL VECTORS

The lentiviral vectors systems consisted of four vector types: pGLV4-GFP, pGag/Pol, pRev, and pVSV-G. Based on a FoxO1 sequence searching from an NCBI reference sequence (NM_001191846.2) and the Zhao et al. study [Zhao et al., 2010], three conservative serine/threonine sites were mutated (Thr→Ala-24, Ser→Ala-256, Ser→Ala-319) to create nucleus-localized FoxO1-coding sequences (3A-FoxO1). The sequence of siRNA targeting FoxO1 (siRNA-FoxO1) was 5′-CCAGCTATAAATGCACATTTA-3′ according to our previous study [Liu et al., 2014]. DNA sequencing confirmed the accurate insertion of target sequences. Next, the pGag/Pol, pRev, and pVSV-G plasmids, together with pGLV4-GFP/3A-FoxO1 or pGLV4-GFP/SiRNA-FoxO1 or pGLV4-GFP plasmids were co-transfected into 293T cells to package 3A-FoxO1 lentiviral vectors (LV-3A-FoxO1), SiRNA-FoxO1(LV-SiNRA-FoxO1) or normal control lentiviral vectors (LV-pGLV4-GFP). After 6 h of culture, the medium was replaced with thorough washing. The supernatants replete with lentiviral vectors were collected after 72 h.

CELL CULTURE AND ESTABLISHMENT OF STABLE FOXO1 OVEREXPRESSION IN MCS

Rat MCs were cultured in normal glucose (5.6 mmol/L)-containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, HEPES buffer, PH 7.2, and penicillin/streptomycin (Gibco, Grand Island, NY). After 4–6 generations of culture, MCs growing in the logarithmic phase were digested and plated at a concentration of 2×10^3 cells/well into 96-well plates. After 24 h of culture, the cells were transfected with lentiviral vectors in serum-free medium with various multiplicities of infection (MOI): 1, 10, or 100. The transfection enhancer Polybrene (Genepharma, Shanghai, PRC) was added into each well at a concentration of 5 μg/mL. The medium was subsequently replaced with complete medium after 24-h incubation with lentivectors. After culturing for an additional 72 h, GFP expression was observed by fluorescence microscopy (IX71, OLYMPUS, Japan), and the positive rate of GFP was assessed by flow cytometer (EPICS XL, Beckman Coulter, CA). The optimal titer was determined based on the transfection efficiency and cytotoxicity of lentiviral vectors. For subsequent experiments, MCs were first cultured in normal glucose conditions and transfected with respective lentivirual vectors. Then, MCs without or with transfection were received respective treatments for 72 h: MCs without transfection cultured in normal glucose (5.6 mmol/L) or high glucose (25 mmol/L) medium served as the normal glucose group (group NG) or high glucose group (group HG); MCs transfected with LV-3A-FoxO1 and treated in high glucose or normal glucose medium served as group HG-3A or group NG-3A; MCs transfected with LV-SiRNA-FoxO1 and cultured in normal glucose medium served as group SiRNA-FoxO1; MCs transfected with LV-pGLV4-GFP and cultured in high glucose medium served as normal control group (group NC). In addition, MCs transfected with LV-pGLV4-GFP and cultured in normal glucose medium were also used to exclude the influence from lentiviral vector. All of the experiments described below were carried out for 3 times.

REAL-TIME POLYMERASE CHAIN REACTION ANALYSIS

After treatments described above, total RNA was extracted from MCs using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed according to the manufacturer's protocol (Toyobo, Osaka, Japan). The expression of target gene was assessed by quantitative real-time PCR using KOD SYBR green master mix (Toyobo) by a sequence detection system (ABI Fast 7500; Applied Biosystems, Foster City, CA). Relative quantification of the target gene was normalized to rat β-actin levels and calculated with the $2^{-\Delta\Delta Ct}$ method. Refer to Table SI for the primer sequences.

IMMUNOFLUORESCENCE

MCs were grown in chamber slides before treatment. After 72 h of incubation, the MCs were fixed by 4% paraformaldehyde solution at room temperature (RT) for 20 min, and permeabolized with 0.25% Triton X-100. After washing with TBS, MCs were blocked with 1% BSA for 30 min, followed by incubation with phospho-Smad3

antibody (1:200) at 4°C overnight. MCs were incubated with Cy3-tagged secondary antibody at room temperature for 1 h, followed by incubation with DAPI for nuclear staining. The distribution and localization of target proteins were visualized by fluorescence microscopy (IX71, OLYMPUS, Japan). The quantification of immunofluorescence was measured by Image-Pro Plus 6.0.

WESTERN BLOTTING

After the aforementioned treatments, total proteins of MCs were extracted by incubation in RIPA buffer (Sangon Biotech, Shanghai, PRC) with 1% protease inhibitor cocktail at 4°C for 30 min. Equal amounts of whole-cell lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to PVDF membrane. The membrane was incubated with 5% BSA for 2 h, followed by hybridization at 4°C overnight with one of the following primary antibodies: anti-FoxO1 (1:1000), anti-phospho-FoxO1 (1:750), anti-Akt (1:750), anti-phospho-Akt (1:500), anti–TGF-β (1:1000), anti-TGF-βRI (1:1000), anti-TGF-βRII (1:1000), anti-Smad3 (1:750), anti–phospho-Smad3 (1:1000). After washing with TBS-Tween 20 (TBST) buffer, the membrane was incubated with HRP-conjugated secondary antibody (1:10,000) for 1 h. The bands were detected by enhanced chemiluminescence substrate (ECL; Thermo Fisher Scientific, Waltham, MA) and exposed to X-ray film. Relative expressions of the target proteins were normalized to GAPDH levels.

ELISA

Conditioned media were collected after 72 h of culture in each group. After centrifuge at speed of 1000*g* for 15 min at 4°C, the supernatant was collected followed by measuring FN and Col I through ELISA kits under the instruction of manufacturer's protocol with three duplicate wells. The optical density of each well was detected by microplate reader. After standard curve was fitted by using Curve Expert 1.3 software, the contents of FN and Col I were calculated.

QUANTIFICATION OF ROS BY USING CM-H2DCFDA PROBE

After culture for 72 h, MCs in each group were collected and rinsed with PBS buffer for three times. MCs were then re-suspended in pre-warmed PBS which contained CM-H2DCFDA probe with concentration of 10 μmol/L. After incubated at 37°C for 20 min, intracellular ROS was detected by using flow cytometer. The mean fluorescence intensity (MFI) was used to represent average level of intracellular ROS in each group.

STATISTICAL ANALYSIS

SPSS 18.0 software (IBM, Endicott, NY) was used to analyze data. Statistical differences between experimental groups were determined by analysis of variance (ANOVA), followed by Bonferroni analysis for comparisons.

RESULTS

EXPRESSION LEVELS OF FOXO1 IN EACH GROUP

First, the optimal MOI for lentiviral vectors was determined. When MOIs of 1, 10, and 100 were used, the corresponding transfection efficiencies were 0.6%, 13.1%, and 78.1%, respectively, as detected by flow cytometry. Since no substantial cell death was observed after transfection, the optimal MOI used was 100 (Fig. S1).

Then, MCs were transfected with LV-3A-FoxO1 or LV-SiRNA-FoxO1 or LV-pGLV4-GFP at optimal MOI. No significant differences were observed in FoxO1 expression at either mRNA or protein level among group NG, group HG and group NC ($P > 0.05$). While FoxO1 expression was significantly elevated after transfection with LV-3A-FoxO1 in either normal glucose or high glucose conditions compared with other groups ($P < 0.05$; Fig. 1A, E, and F). In contrast, expression of FoxO1 obviously decreased in group SiRNA-FoxO1 ($P < 0.05$; Fig. 1A, E, and F). These results confirmed the transfection efficiency of LV-3A-FoxO1 and LV-SiRNA-FoxO1. Meanwhile, no significant differences of all indices were observed between groups HG and NC (Figs. 1–4), as well as MCs transfected with LV-pGLV4-GFP compared to group NG (data not shown), which excluded the negative effect of lentivirial vectors.

EFFECT OF FOXO1 IN EACH GROUP

Accounting on Akt-mediated phosphorylation on FoxO1 is the most important regulation of its bioactivity, total Akt, p-Akt, and p-FoxO1 levels were assessed. The protein level of p-FoxO1 in group HG increased significantly compared with group NG, which was in consistent with the change of p-Akt ($P < 0.05$, Fig. 1B, D, and E). Overexpression of 3A-FoxO1 decreased the protein level of p-Akt and p-FoxO1 in HG-3A group compared with HG and NC groups, and the ratio of p-FoxO1/FoxO1 was significantly reduced ($P < 0.05$ Fig. 1B–E); whereas, MCs in group NG-3A didn't further decrease the level of p-Akt and p-FoxO1 compared with group NG ($P > 0.05$ Fig. 1B, D, and E). In contrast, the level of p-FoxO1 was decreased and the level of p-Akt was increased in group SiRNA-FoxO1 ($P < 0.05$ Fig. 1B–E).

Next, the expression of catalase and SOD2, two target genes of FoxO1 involving in anti-oxidative stress, was detected in each group. Total intracellular ROS were also measured. Both catalase and SOD2 mRNA levels were reduced in group HG compared with group NG, while overexpression of 3A-FoxO1 significantly elevated the expression of catalase and SOD2 in either group NG-3A or group HG-3A compared with other groups ($P < 0.05$ Fig. 1G and H). Moreover, high glucose increased the mean fluorescence intensity (MFI) of ROS in MCs, while overexpression of 3A-FoxO1 in high glucose decreased ROS level ($P < 0.05$ Fig. 1I). In group SiRNA-FoxO1, the expressions of these proteins were decreased which was similar to MCs in group HG-3A; furthermore, significant increase of ROS level was observed in group SiRNA-FoxO1 compared to group NG ($P < 0.05$ Fig. 1G–I). MCs in NG-3A group expressed lowest ROS level among all groups ($P < 0.05$ Fig. 1I).

EXPRESSION LEVELS OF FN AND Col I IN EACH GROUP

FN and Col I are two major constituents of the ECM, which accumulate in the mesangium of glomerulus and aggravate existing diabetic nephropathy. Thus, we were interested in determining whether FoxO1 was associated with FN and Col I synthesis in MCs. The mRNA and protein levels of FN and Col I

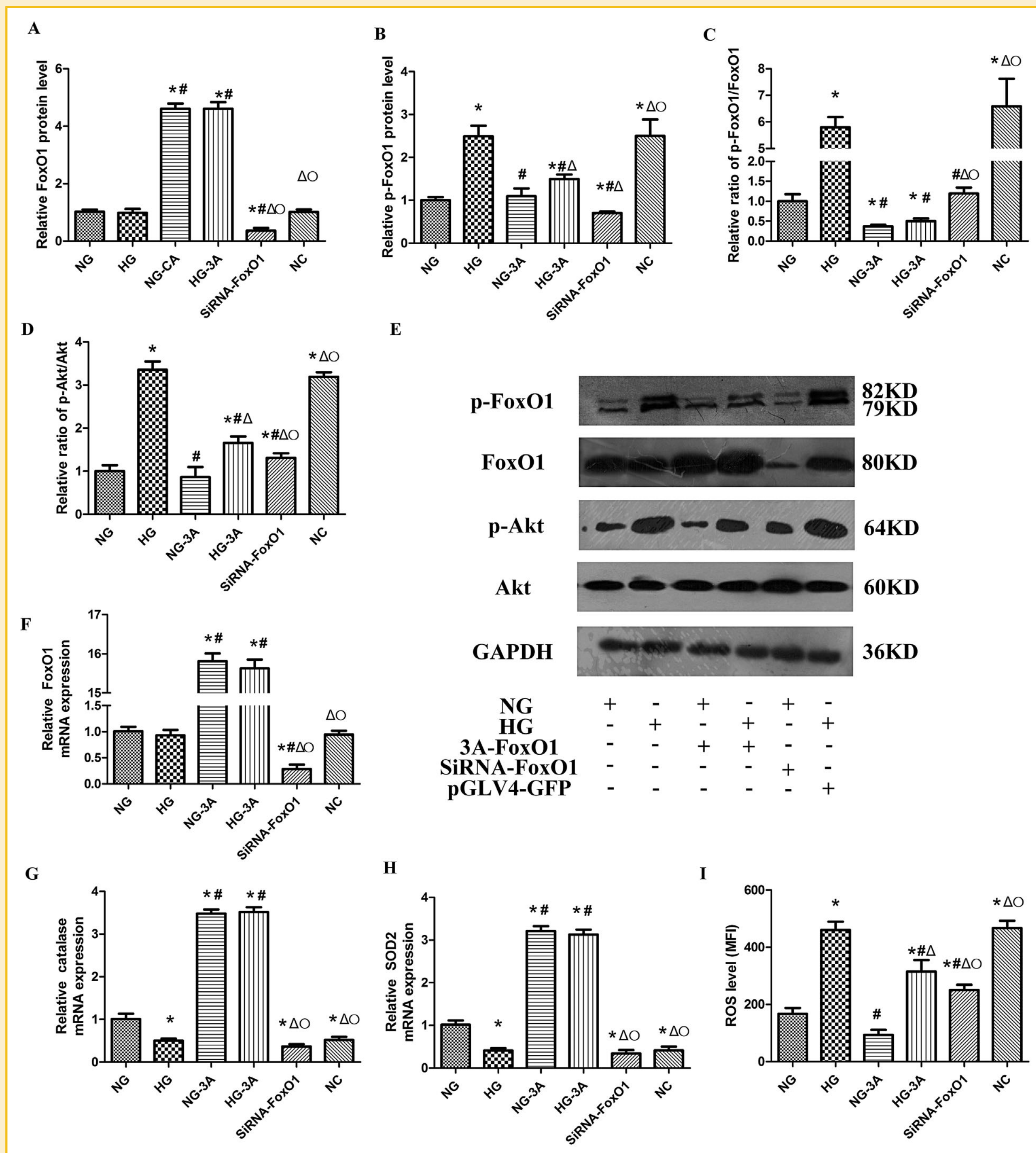

Fig. 1. The level of forkhead transcription factor O1 (FoxO1), phospho(p)-FoxO1, Akt, p-Akt, catalase, SOD2, and intracellular ROS in each group. A: relative FoxO1 protein level; B: relative p-FoxO1 protein level; C: the ratio of p-FoxO1/FoxO1; D: the ratio of p-Akt/Akt; E: the protein levels of FoxO1, p-FoxO1, Akt and p-Akt detected by western blotting; F: FoxO1 mRNA level; G: catalase mRNA level; H: SOD2 mRNA level; I: MFI of intracellular ROS level. The data are displayed as mean ± SEM versus group NG, $^{*}P < 0.05$; versus group HG, $^{\#}P < 0.05$; versus group NG-3A, $^{\Delta}P < 0.05$; versus group HG-3A, $^{\circ}P < 0.05$

were both significantly up-regulated in group HG compared with group NG ($P < 0.05$ Fig. 2A–D). As expected, overexpression LV-3A-FoxO1 attenuated the increase of FN and Col I expression level which was induced by high glucose ($P < 0.05$ Fig. 2A–D). No statistically significant differences were observed in Col I mRNA and protein level between groups HG-3A, NG-3A, and NG ($P > 0.05$ Fig. 2B and D), unlike FN mRNA and protein level, which remained significantly higher for group HG-3A than group NG and group NG-3A ($P < 0.05$; Fig. 2A and C). In addition, down-regulation of FoxO1 by LV-SiRNA-FoxO1 increased the expression

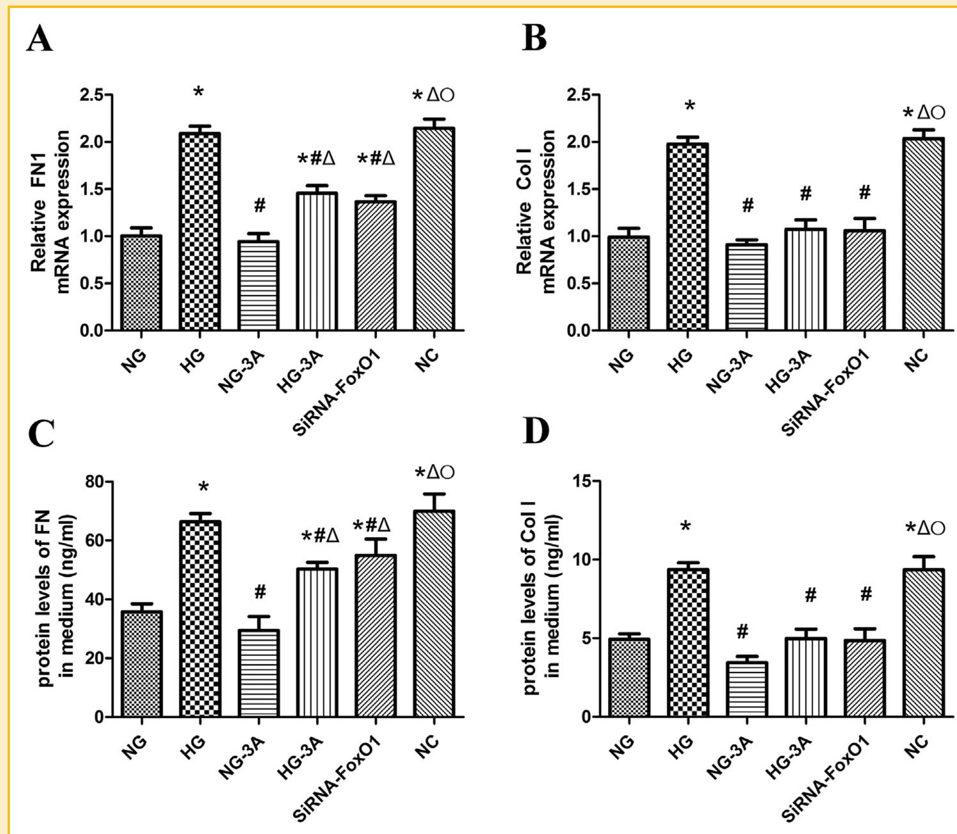

Fig. 2. The expression level of fibronectin (FN) and collagen I (Col I) in each group. A: FN mRNA level; B: Col I mRNA level; C: protein level of FN detected by ELISA; D: protein level of Col I detected by ELISA. The data are displayed as mean ± SEM versus group NG, $^{*}P < 0.05$; versus group HG, $^{\#}P < 0.05$; versus group NG-3A, $^{\Delta}P < 0.05$; versus group HG-3A, $^{\circ}P < 0.05$.

of FN in normal glucose conditions ($P < 0.05$ Fig. 2A and C); however, the change of Col I levels between NG and NG-3A showed no significant difference ($P > 0.05$ Fig. 2B and D).

EXPRESSION LEVELS OF TGF-β1 AND TGF-β RECEPTORS IN EACH GROUP

The TGF-β/Smad pathway plays an essential role in regulating ECM secretion, so we investigated whether 3A-FoxO1 affects the activation of this signaling pathway. Relative TGF-β1 mRNA level was significantly elevated in group HG compared with group NG ($P < 0.05$), while relative TGF-β1 mRNA level in group HG-3A significantly decreased compared with group HG ($P < 0.05$; Fig. 3A). MCs in group SiRNA-FoxO1 expressed higher TGF-β1 mRNA level than group NG ($P < 0.05$ Fig. 3A). Comparable results were observed in TGF-β1 protein expression from western blotting analysis ($P < 0.05$; Fig. 3D–F).

Mature TGF-β1 can form complexes with TGF-βRI and TGF-βRII, thereby initiating signal transduction from membrane to cytoplasm. Next, we detected the expression level of type I (ALK-5) and type II TGF-β receptors. Real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis showed that mRNA and protein levels of TGF-βRI and TGF-βRII were both elevated under high glucose conditions ($P < 0.05$ Fig. 3B, C, G, and H). LV-3A-FoxO1 transfection significantly down-regulated mRNA and protein levels of TGF-βRI and TGF-βRII, while LV-SiRNA-FoxO1 transfection exhibited opposite effects ($P < 0.05$; Fig. 3B, C, G, and H).

EXPRESSION LEVELS OF Smad3 AND p-Smad3 IN EACH GROUP

Smad3 is a TGF-β receptor-specific transcription factor that is recruited and phosphorylated by activated TGF-βRI, thereby transducing TGF-β-mediated signaling from the cytoplasm to the nucleus. Therefore, we investigated whether FoxO1 was involved in regulating Smad3 expression. Interestingly, Smad3 mRNA and protein levels were elevated in group HG compared with group NG, NG-3A, and SiRNA-FoxO1 ($P < 0.05$), while no significant difference in either Smad3 mRNA or protein level was observed among group HG-3A, HG, and NC ($P > 0.05$ Fig. 4A, C, and E). The results indicated that the expression level of Smad3 was directly regulated by high glucose conditions which may be independent of the effect of FoxO1.

TGF-β receptor-induced phosphorylation of Smad3 promotes its association with the co-mediator Smad4, followed by translocation into the nucleus and subsequent binding to the Smad binding element (SBE) in the target gene for activation. Therefore, we examined p-Smad3 protein level in each group. Compared to group NG, p-Smad level was elevated in group HG and NC, as well

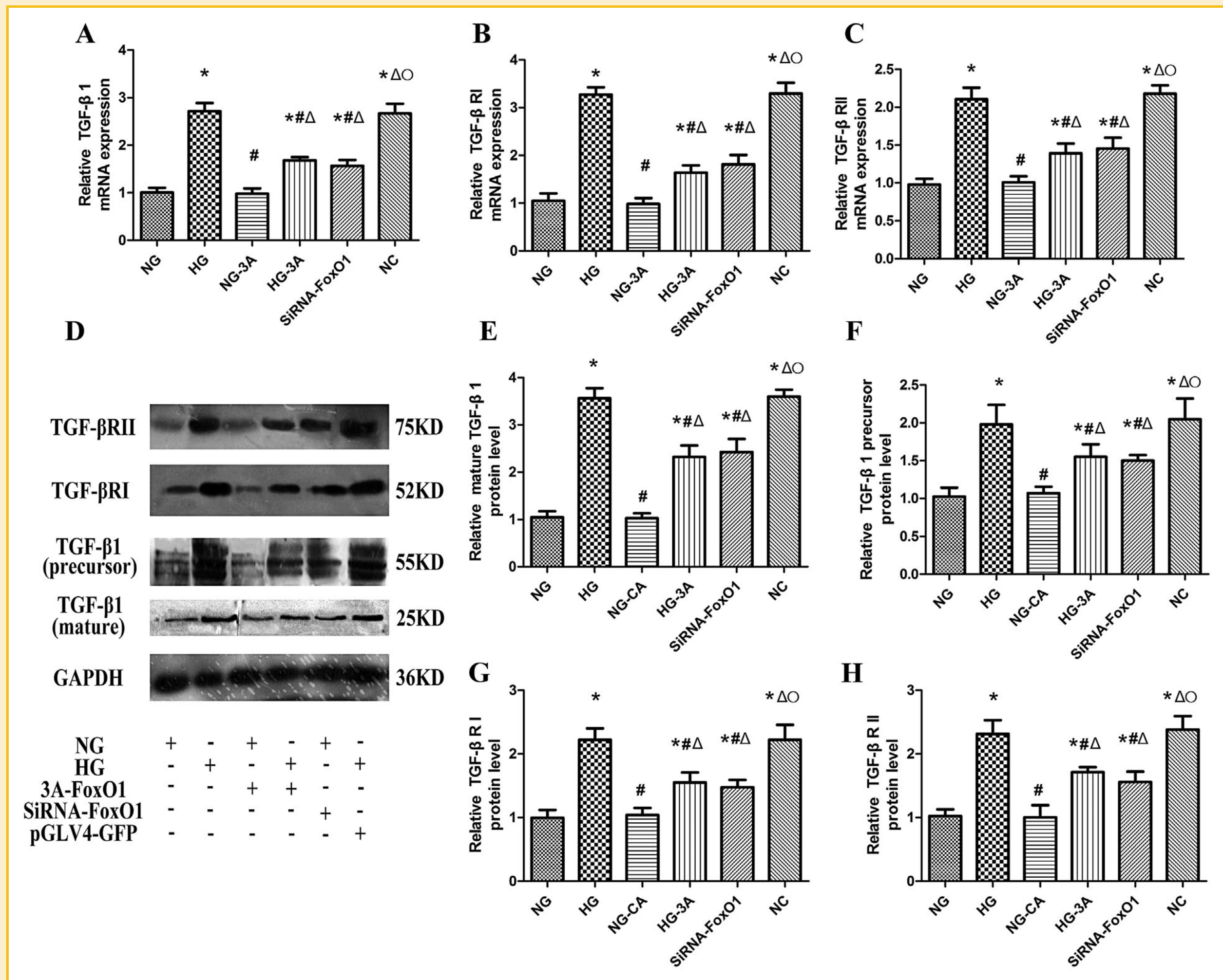

Fig. 3. The expression level of transforming growth factor (TGF)-β1, transforming growth factor-β receptor I (TGF-βRI), and TGF-βRII in each group. A: TGF-β1 mRNA level; B: TGF-βRI mRNA level; C: TGF-βRII mRNA level; D: results from western blotting; E: relative protein level of TGF-β1 precursor; F: relative protein level of mature TGF-β1; G: relative protein level of TGF-βRI; H: relative protein level of TGF-βRII. The data are displayed as mean ± SEM versus group NG, $^{*}P < 0.05$; versus group HG, $^{\#}P < 0.05$; versus group NG-3A, $^{\Delta}P < 0.05$; versus group HG-3A, $^{\circ}P < 0.05$.

as in group SiRNA-FoxO1 ($P < 0.05$). By contrast, cells transfected with LV-3A-FoxO1 displayed attenuated Smad3 phosphorylation under high glucose conditions ($P < 0.05$; Fig. 4A and D). But no difference was observed between group NG and group NG-3A ($P > 0.05$).

INTRACELLULAR LOCALIZATION OF p-Smad3 IN EACH GROUP

Continuous nucleocytoplasmic shuttling of Smad3 has proven to be essential for signal transduction in the TGF-β/Smad pathway. Therefore, we investigated the intracellular localization of p-Smad3 in each group. Immunofluorescence quantification and analysis indicated that either high glucose conditions or transfection with LV-SiRNA-FoxO1 increased p-Smad3 staining in the nucleus, consistent with an overall increase in p-Smad3 expression ($P < 0.05$, Fig. 4B and F). By contrast, overexpression of 3A-FoxO1 attenuated MFI of p-Smad3 in nucleus of MCs under high glucose, in concert with faintly observed overall immunofluorescence ($P < 0.05$, Fig. 4B and F), indicating lower basal level of p-Smad and diminished transcriptional activity. While the intensity of fluorescence in MCs in NG-3A group showed no difference from which in NG group (Fig. 4B and F).

DISCUSSION

In the present study, we have uncovered a novel role for FoxO1 in regulating the secretion of ECM proteins in MCs. Excessive deposition of ECM protein in the mesangium and basement membrane plays a pivotal role in promoting glomerulosclerosis during early-stage DN [Kolset et al., 2012]. FoxO1 transcription factor is implicated in controlling protein synthesis and degradation, so it may be associated with modulating the

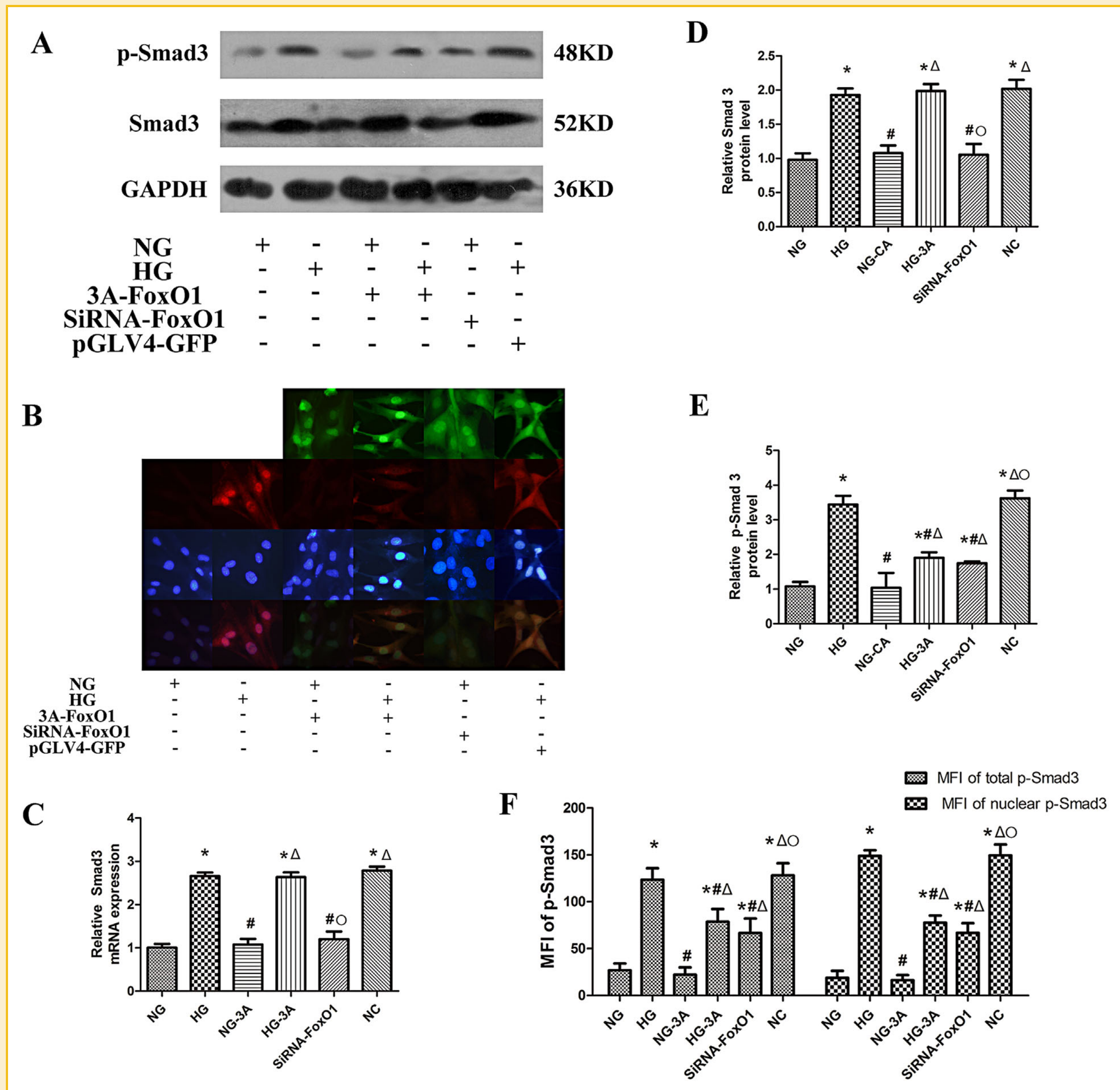

Fig. 4. The expression of Smad3 and p-Smad3 in each group. A: results from western blotting; B: The immunofluorescence result of p-Smad3. The expression level of green fluorescent protein (GFP) in MCs transfected with lentiviral vectors are displayed (GFP, top line). The fixed MCs were incubated with anti-p-Smad3 antibody, followed by Cy3-tagged secondary antibody (p-Smad3, the second line), and then counterstained with DAPI to display the nucleus after incubation with antibody (DAPI, the third line). The merged images are displayed in the last line (Merge). Original magnification, 400×; C: Smad3 mRNA level; D: relative protein level of Smad3; E: relative protein level of p-Smad3; F: MFI of total p-Smad3 (left) and nuclear p-Smad3 (right). The data are displayed as mean ± SEM versus group NG, $^{*}P < 0.05$; versus group HG, $^{\#}P < 0.05$; versus group NG-3A, $^{\Delta}P < 0.05$; versus group HG-3A, $^{\circ}P < 0.05$.

expressions of ECM proteins [Rached et al., 2010]. Previous studies demonstrated that HG incubation can active PI3K/Akt pathway thereby sequester FoxO1 in cytoplasm and attenuate its bio-activity by phosphorylating three conserved sites (Thr-24, Ser-256, Ser-319), whereas phosphorylation by Jun N-terminal kinase (JNK) or mammalian Ste20-like kinase-1 (MST1) may increase FoxO transcriptional activity [Tang et al., 1999; Kawamori et al., 2006; Lehtinen et al., 2006]. Although both mRNA and protein levels of FoxO1 were not altered in MCs after exposure to high glucose, p-FoxO1 level was significantly elevated in association with the up-regulation of p-Akt levels, indicating that high glucose-induced activation of Akt might be involved in the inactivation of FoxO1. In the meanwhile, FN and Col I expression levels significantly elevated under high glucose conditions, suggesting a possible link between inactivation of FoxO1 by PI3K/Akt and secretion of ECM protein in MCs.

Therefore, we synthesized nucleus-localized FoxO1-coding sequences to antagonize phosphorylation of FoxO1 by high glucose-induced PI3K/Akt activation [Buteau et al., 2007; Zhao et al., 2010], and SiRNA-targeting FoxO1 for further confirmation of its effect. Lentiviral vectors containing 3A-FoxO1 or SiRNA-FoxO1 sequences were constructed to ensure high transfection efficiency. As expected, FoxO1 mRNA and protein levels in MCs were both dramatically increased after transfection with LV-3A-FoxO1, while decreased after transfection with LV-SiRNA-FoxO1, suggesting that the regulation of FoxO1 by lentiviral vectors occurred at both transcriptional and translational levels. Then we observed the down-regulation of both p-Akt and p-FoxO1 protein levels after overexpression of 3A-FoxO1, which is similar to the results from recent study that FoxO1 control a positive feed-back loop for sustained Akt activation in response to high glucose [Das et al., 2014]. Concomitantly, the expression levels of two primary ECM constituents-FN and Col I-decreased after transfection, associated with an increase in FoxO1 bioactivity. In contrast, specific degradation of FoxO1 by LV-siRNA-FoxO1 in MCs led to the up-regulation of p-Akt and FN, further confirming the effects of 3A-FoxO1 on inhibiting high-glucose-stimulated Akt activation and FN protein secretion in MCs. While the Col I level didn't change after transfection with siRNA-FoxO1, indicating different regulation mechanism of FoxO1 on ECM secretion.

Next, we investigated the possible mechanism by which FoxO1 inhibited ECM secretion in MCs. Previous studies reported that activated TGF-β/Smad pathway is highly associated with ECM protein secretion in MCs [Li et al., 2003; Ghosh and Abboud, 2004; Medina et al., 2011]. The active form of mature TGF-β1 which is cleaved from latent TGF-β1 in ECM can trigger heteromeric complex formation with TGF-βRII and TGF-βRI, thereby initiating TGF-β pathway [Shi and Massague, 2003; Dallas et al., 2005]. Consistent with previous studies [Ha et al., 2001; Li et al., 2008], our data suggested that the TGF-β1 mRNA and protein levels were significantly increased in MCs under high glucose conditions, in concert with an increase in both TGF-βRI and TGF-βRII expression levels. By contrast, overexpression of 3A-FoxO1 attenuated the expression levels of both latent TGF-β1 and mature TGF-β1, indicating an attenuation in the synthesis and transformation of TGF-β1. Concomitantly, levels of both TGF-βRI and TGF-βRII were decreased as well. Moreover, elevations of TGF-β1 and its receptors were observed in MCs transfected with LV-SiRNA-FoxO1 and cultured in normal glucose, which in turn affirmed the role of FoxO1 in weakening the initiation of TGF-β signaling. Our findings suggested that the process of TGF-β transmembrane signaling was attenuated by FoxO1 expression.

The downstream transcription factor Smad3 is required for signal transduction in the TGF-β pathway. Phosphorylation of Smad3 by active TGF-βRI leads to complex formation with Smad4, followed by nuclear translocation [Shi and Massague, 2003; Massague, 2012]. After releasing from the complex, Smad3 binds to the promoter, associates with other transcription factors, and initiates the transcription of target genes [Das et al., 2008; Lu et al., 2012]. Our results illustrated that both 3A-FoxO1 and SiRNA-FoxO1 did not affect Smad3 expression levels, whereas high-glucose-induced elevation of p-Smad3 protein was decreased after overexpression of 3A-FoxO1. Furthermore, the immunofluorescence analysis demonstrated that an up-regulation of the phosphorylate form of Smad3 was enhanced under high glucose conditions accompanied by an increase of p-Smad3 protein expression, whereas overexpression of 3A-FoxO1 attenuated the nuclear translocation of p-Smad3. Similarly to MCs cultured in high glucose, knocking-down of FoxO1 by LV-SiRNA-FoxO1 also contributes to the phosphorylation of Smad3 in MCs under normal glucose. Taken together, these data demonstrate the role of FoxO1 in inhibiting phosphorylation and nuclear translocation of Smad3, which contributes to the attenuation of FN and Col I mRNA expression in MCs.

Although the link between FoxO1 and TGF-β/Smad pathway have been established as mentioned above, the underlying mechanism is not clear. Previous studies demonstrated that reactive oxygen species (ROS) plays pivotal roles in the pathogenesis of DN [Kowluru and Kanwar, 2009; Arora and Singh, 2014; Zhang et al., 2014]. High glucose-induced excessive production of ROS fortifies the activation of both TGF-β/Smad pathway, while the activation of this pathway in turn intensifies the generation of ROS [Sohn et al., 2012; Hagler et al., 2013; Lu et al., 2013]. In consideration of anti-oxidative stress which is one of the most important effects of FoxO1 [Subauste and Burant, 2007; Storz, 2011], it might be possible that FoxO1-mediated anti-oxidative effect may interfere with the activation of TGF-β/Smad pathway. Catalase and SOD2, two major target genes of FoxO1 involving in anti-oxidative, were significantly up-regulated after overexpression of 3A-FoxO1, which was in concert with the decrease of ROS level and attenuation of TGF-β/Smad pathway in MCs in high glucose. Although cultured in normal glucose conditions, MCs transfected with LV-SiRNA-FoxO1 exhibited attenuated the expressions of catalase and SOD2 accompanied with elevated activation of both ROS level and TGF-β/Smad pathway, which was similar to those 3A-FoxO1 MCs cultured in high glucose conditions. Therefore, we proposed that FoxO1 may attenuate the activation of TGF-β/Smad pathway in MCs through anti-oxidative effect under high glucose conditions. Interestingly, although the expression of anti-oxidative enzyme was increased and the level of ROS decreased, incubation of cells with normal glucose along with 3A-FoxO1 expression did not further affect the activation of TGF-β/Smad pathway and the synthesis of ECM proteins, indicating that lower level of ROS in homeostasis may not trigger the effect of FoxO1 on ECM proteins synthesis.

Apart from activating the TGF-β/Smad pathway, TGF-β1 also intersects with non-Smad pathway, including GTPases, Rho, Rac, and Ras-Erk-MAPK pathway, as well as the PI3K/Akt pathway [Bakin et al., 2000; Edlund et al., 2005; Moustakas and Heldin, 2005; Samarakoon and Higgins, 2008]. Interestingly, it is reported that activation of Akt is also implicated in TGF-β-induced biological effects [Bakin et al., 2000; Runyan et al., 2004; Das et al., 2008]. Moreover, the interaction between PI3K/Akt and TGF-β pathway may aggravate the inactivation of FoxO [Kato et al., 2006; Lu et al., 2013]. The present study illustrated the role of FoxO1 in attenuating activation of Akt, which may also contribute to the inactivation of TGF-β signaling. Current researches have identified that FoxO1 interacts with PI3K/Akt/mTORC1 pathway or TIMP3/STAT1 to ameliorate the pathogenesis of DN, which are supplements to our

research [Fiorentino et al., 2013; Das et al., 2014]. Using hepatocytes and endothelial cells, two laboratories have suggested that FoxO1 can suppress TGF-β1/Smad3-induced transcription of Plasminogen activator inhibitor 1(PAI-1) by interfering with Smad3 activity on the promoter of target genes [Fujita et al., 2006; Jung et al., 2009]. However, other investigators have indicated that FoxO3a-another member of FoxO family plays a crucial mediatory role in the TGF-β1 signaling pathway leading to apoptosis in FaO rat hepatoma cells [Kim, 2008]; in addition, FoxO1 is integrated with Smad proteins in the control of cell proliferation in neuroepithelium and keratinocytes [Seoane et al., 2004; Gomis et al., 2006]. These results, together with our findings, suggest that the interaction between FoxO1 and the TGF-β pathway may be cell-specific, and be relevant to the type and function of the particular target gene.

In summary, the present study demonstrated that high glucose conditions increased the expression of fibronectin and collagen I by activating PI3K/Akt and TGF-β/Smad3 pathway in mesangial cells, while lentiviral vector-mediated overexpression of nucleus-localized FoxO1 attenuated this effect by up-regulation anti-oxidative target genes. Our data suggest FoxO1-mediated anti-oxidative effect may play a vital role in attenuating ECM protein secretion and alleviating the pathological changes associated with diabetic nephropathy. However, the effects of FoxO1 in association with ECM secretion remain to be confirmed in vivo.

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