Original Article

DOI: 10.5582/bst.2016.01224

Knocking down TCF8 inhibits high glucose- and angiotensin II-induced epithelial to mesenchymal transition in podocytes

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Summary **Epithelial to mesenchymal transition (EMT) is a physiological phenomenon in mammalian embryogenesis by which epithelial cells become mesenchymal stem cells. Studies have indicated that an inappropriate EMT plays a key role in a variety of pathogenic processes such as embryonic development and tumor metastasis. Moreover, recent studies have indicated EMT also plays an important role in renal fibrosis. In the current study, glucose and angiotensin II promoted EMT in podocytes as well as changes in the cellular morphology of podocytes. A high concentration of glucose and angiotensin II also promoted podocyte movement and migration. Moreover, a high concentration of glucose and angiotensin II promoted TCF8 expression. Inhibiting TCF8 expression with siRNA reversed EMT in podocytes in the presence of a high concentration of glucose and angiotensin. Inhibiting TCF8 expression also reversed changes in cellular morphology and podocyte movement and migration. Therefore, glucose and angiotensin II may promote EMT in podocytes via TCF8.**

Keywords: Epithelial to mesenchymal transition (EMT), podocyte, glucose, angiotensin II, TCF8

1. Introduction

In the kidneys, podocytes are attached to the outside of the glomerular basement membrane. The glomerular basement membrane plays an important role in maintaining the normal structure and function of the glomerulus *via* the actin cytoskeleton of podocytes and specific expression of protein molecules (*1*). Nephrin, WT1, and synaptopodin are phenotypic markers of mature podocytes (*2*). Podocytes have long processes that interdigitate and that are separated by very narrow spaces (30-40 nm) that are bridged by a membrane called the slit diaphragm (*3*). This diaphragm allows water and small solute molecules to pass through but it filters out plasma proteins and other macromolecules, and this property is the main determinant of the permeability of the renal glomerular filtration barrier (*1*). Therefore, podocytes play an important role in protecting the integrity of the glomerular filtration barrier, adjusting the ultrafiltration coefficient, and maintaining the normal structure of the glomerular basement membrane (*4*). Ultrastructural changes in podocytes and reduced expression of associated molecules is closely related to renal impairment and can lead to glomerulosclerosis and renal interstitial fibrosis (*5*).

The epithelial-mesenchymal transition (EMT) is a process by which polarized epithelial cells undergo numerous biochemical changes to obtain a mesenchymal cell phenotype (*6*). EMT is also involved in tumor migration and invasion (*7*). Podocytes are epithelial cells, so when they are affected by different conditions (such as TGF-β, a high concentration of glucose, doxorubicin, or some other stimuli), EMT may occur (*8-10*). When EMT occurs, epithelial phenotypic markers such as E-cadherin, P-cadherin, nephrin, podocalyxin, and synaptopodin are downregulated, and mesenchymal phenotypic markers

Released online in J-STAGE as advance publication January 22, 2017.

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such as desmin, FSP-1, MMP-9, ILK, fibronectin, vimentin, α-SMA, collagen I, and snail are upregulated (*11*). EMT is considered to be the key factor for an impaired kidney producing myofibroblasts in renal fibrosis, which may lead to renal interstitial fibrosis (*12*). Podocytes, which are also known as glomerular visceral epithelial cells, are cells affected by different types of injury. EMT in podocytes will result in damage to the glomerular filtration barrier and proteinuria; in severe cases, it may lead to podocyte detachment from the glomerular basement membrane and apoptosis, causing a decrease in the number of podocytes, thereby aggravating proteinuria and glomerular sclerosis (*13,14*). EMT in podocytes is a key starting point for studying the mechanisms by which proteinuria and renal fibrosis develop (*15*).

The aim of the current study was to examine EMT in podocytes in the presence of a high concentration of glucose and angiotensin II. This study examined the effects of a high concentration of glucose and angiotensin II on EMT and the expression of TCF8 in podocytes. This study also examined the effects of downregulating TCF8 on EMT in podocytes.

2. Materials and Methods

2.1. *Cell culture and reagents*

The podocyte line used in this study was donated by Prof. Ding Jie (Peking University First Hospital, Beijing, China). E-cadherin, α-catenin, N-cadherin, vimentin, TCF8, and β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. *qRT-PCR*

Total RNA was extracted from cell lines using the TRIZOL reagent (Invitrogen). Reverse transcription (RT) was performed using the Thermoscript RT System (Invitrogen). Hotstart PCR conditions were as follows: 45 s at 94°C, 30 s at 55°C, and 1 min at 72°C for 28-30 cycles (for TCF8) or 26 cycles (for glyceraldehyde 3-phosphate dehydrogenase (GAPDH)). The primers used in the study were: TCF8: sense 5'-GCACAACCAAGTGCAGAAGA-3' and antisense 5'-CATT TGCAGATTGAGGCTGA-3'; GAPDH: sense 5'-TGCCTCCTGCACCACCAACT-3' and antisense 5'-CCCGTTCAGCTCAGGGATGA-3'.

2.3. *Plasmid construction and transfection*

For TCF8 RNA interference, control and TCF8 shRNA plasmids (#1: CCGGGCTGCCAATAAGCAAACGAT TCTCGAGAATCGTTTGCTTATTGGCAGCTTTTT; #2: CCGGGCTGTTGTTCTGCCAACAGTTCTCGA GAACTGTTGGCAGAACAACAGCTTTTT; #3: CC GGCGGCGCAATAACGTTACAAATCTCGAGATT TGTAACGTTATTGCGCCGTTTTT) were purchased from Sigma-Aldrich Biotechnology (Sigma-Aldrich, CA, USA) and were used to transfect podocytes to establish a cell line with knocked-down TCF8. The transfection efficiency of TCF8 was verified with Western blotting and quantitative reverse transcription PCR (qRT-PCR) analyses.

2.4. *Western blot assay*

Equal amounts of protein were separated using SDS polyacrylamide gels and were electrotransferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were immunoblotted overnight at 4°C with primary antibodies, followed by their respective secondary antibodies. β-actin was used as the loading control.

2.5. *Cell invasion and motility assay*

Cell invasion was measured in Matrigel (Sigma, St. Louis, MO, USA)-coated Transwell inserts (6.5 mm, Costar, NY, USA) containing polycarbonate filters with 8-μm pores as previously described. The inserts were coated with 50 μL of 1 mg/mL Matrigel matrix in accordance with the manufacturer's recommendations. 2×10^5 cells in 200 μL of serum-free medium were plated in the upper chamber, and 600 μL of medium with 10% fatal bovine serum was added to the lower well. After incubation for 24 h, top cells were removed and bottom cells were counted. Cells that migrated to the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. For each membrane, five random fields were counted at ×10 magnification. The mean was calculated and data were expressed as the mean ± S.D. from three independent experiments done in triplicate. Motility assays were similar to invasion assays with Matrigel except that the Transwell inserts were not coated with Matrigel.

3. Results

3.1. *A high concentration of glucose and angiotensin II promoted EMT in podocytes*

Previous studies reported that a high concentration of glucose and angiotensin II may promote EMT in kidney epithelial cells (*16,17*). The current study examined whether those substances would have the same effect on podocytes. Podocytes were treated with 0, 10, 20, and 30 mM of glucose. As shown in Figures 1A and 1B, the expression of the epithelial marker molecules E-cadherin and α-catenin decreased with an increase in the glucose concentration. As shown in Figures 1C and 1D, the expression of the mesenchymal marker molecules N-cadherin and vimentin increased with an increase in the glucose concentration. Podocytes were then treated

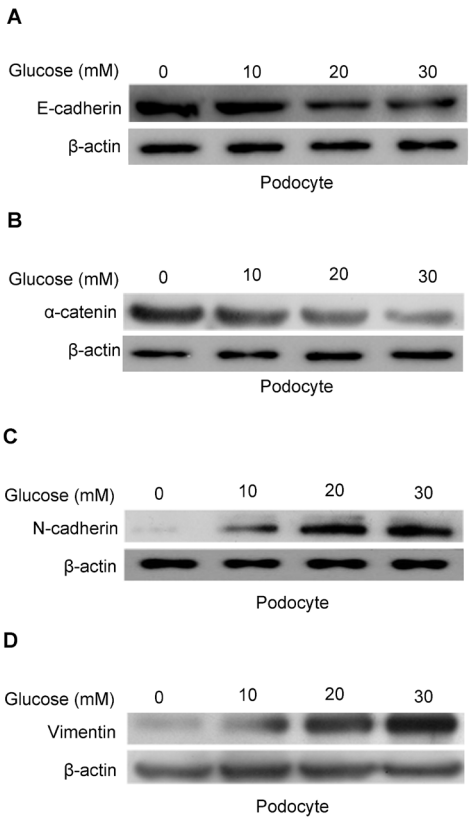

Figure 1. A high concentration of glucose inhibited the expression of epithelial biomarkers (E-cadherin and α-catenin) and promoted the expression of mesenchymal biomarkers (N-cadherin and vimentin) in podocytes. (A) Podocytes were incubated with 0-30 mM of glucose for 24 h and the expression of E-cadherin was measured with Western blotting. **(B)** Podocytes were incubated with 0-30 mM of glucose for 24 h and the expression of α-catenin was measured with Western blotting. **(C)** Podocytes were incubated with 0-30 mM of glucose for 24 h and the expression of N-cadherin was measured with Western blotting. **(D)** Podocytes were incubated with 0-30 mM of glucose for 24 h and the expression of vimentin was measured with Western blotting.

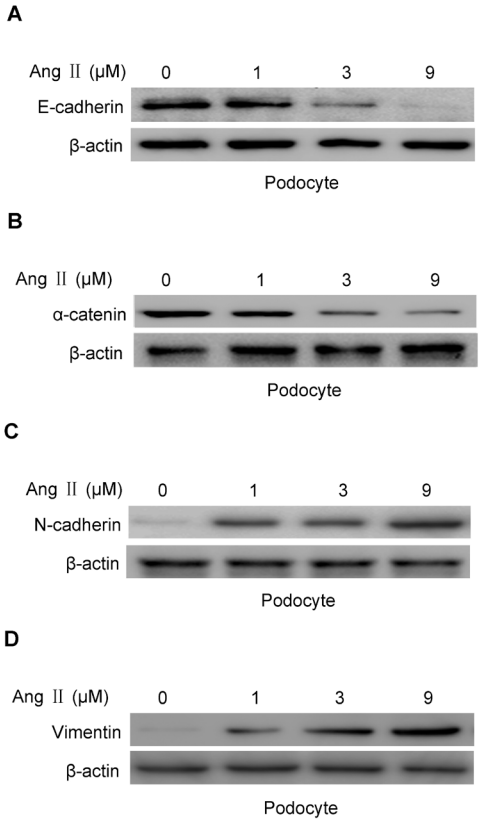

Figure 2. Angiotensin II inhibited the expression of epithelial biomarkers (E-cadherin and α-catenin) and promoted the expression of mesenchymal biomarkers (N-cadherin and vimentin) in podocytes. (A) Podocytes were incubated with 0-9 μM of angiotensin II for 24 h and the expression of E-cadherin was measured with Western blotting. **(B)** Podocytes were incubated with 0-9 μM of angiotensin II for 24 h and the expression of α-catenin was measured with Western blotting. **(C)** Podocytes were incubated with 0-9 μM of angiotensin II for 24 h and the expression of N-cadherin was measured with Western blotting. **(D)** Podocytes were incubated with 0-9 μM of angiotensin II for 24 h and the expression of vimentin was measured with Western blotting.

with 0, 3, 6, and 9 μM of angiotensin II. As shown in Figures 2A and 2B, the expression of the epithelial marker molecules E-cadherin and α-catenin decreased with an increase in the angiotensin II concentration. As shown in Figures 2C and 2D, the expression of the mesenchymal marker molecules N-cadherin and vimentin increased with an increase in the angiotensin II concentration. Results indicated that a high concentration of glucose and angiotensin II promoted EMT in podocytes.

3.2. *A high concentration of glucose and angiotensin II changed podocyte morphology and promoted podocyte migration and invasion*

The current study examined the effects of a high concentration of glucose and angiotensin on podocytes. As shown in Figures 3A and 3B, podocytes displayed morphological changes when stimulated with 30 mM of glucose or 9 μM of angiotensin II. As shown in Figures 3C-3F, a high concentration of glucose and angiotensin promoted podocyte cell migration and invasion. The number of migrating and invading podocytes in the presence of 30 mM of glucose or 9 μM of angiotensin II was about twice that in the presence of 0 mM of glucose or angiotensin II.

3.3. *A high concentration of glucose and angiotensin II promoted TCF8 expression*

TCF8 is a transcription factor that plays an important role in EMT (*6*). The current study examined whether a high concentration of glucose and angiotensin II affects TCF8. Podocytes were treated with 0, 10, 20, and 30 mM of glucose. As shown in Figure 4A, the expression of TCF8 increased with an increase in the glucose concentration. Podocytes were then treated with 0, 3, 6, and 9 μM of angiotensin II. As shown in Figure 4B, the expression of TCF8 increased with an increase in the angiotensin II concentration. Results indicated that a high concentration of glucose and angiotensin II promoted TCF8 expression. TCF8 expression was

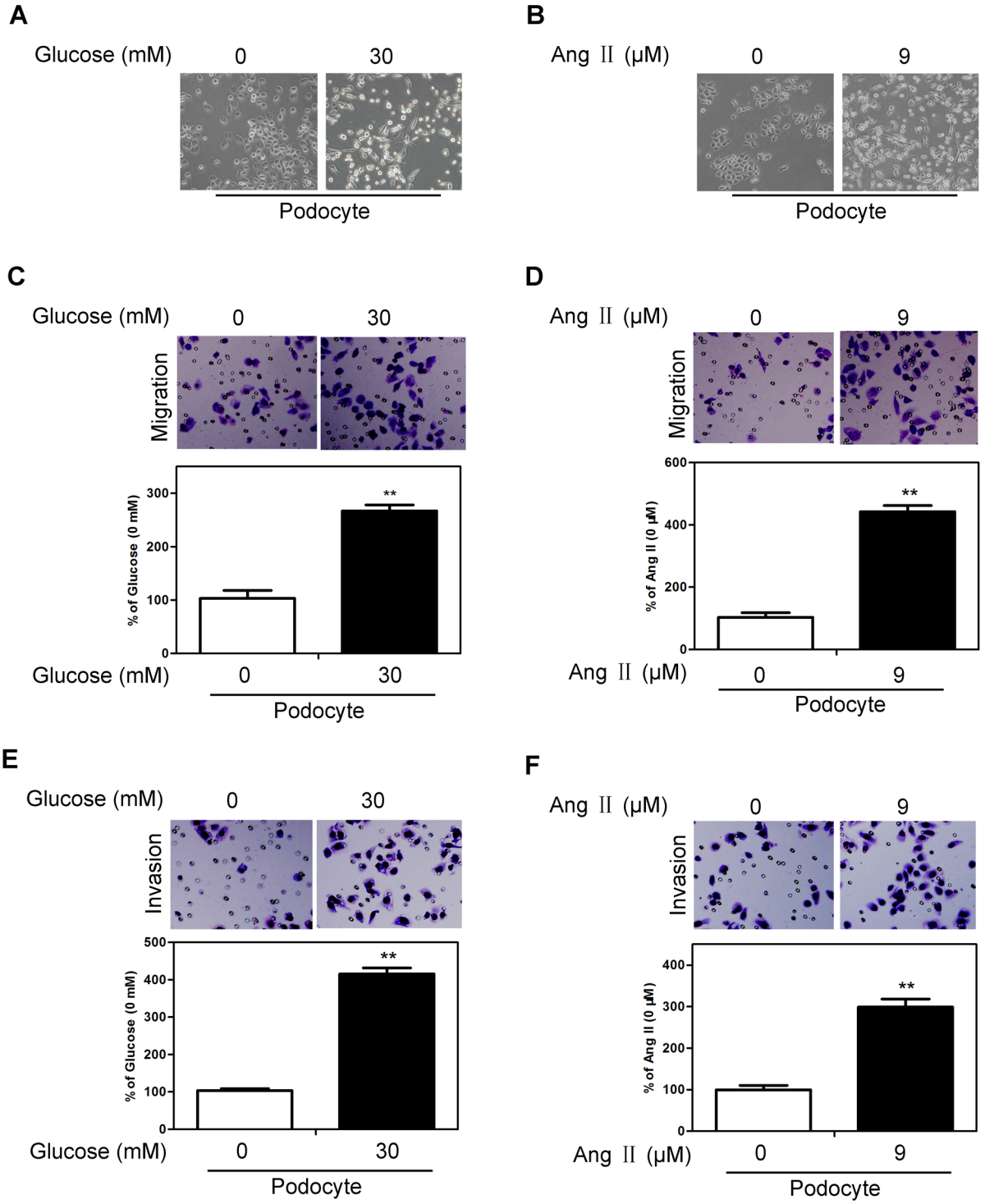

Figure 3. A high concentration of glucose or angiotensin II promoted changes in the cell morphology of podocytes as well as podocyte migration and invasion. (A) Changes in the cell morphology of podocytes in the presence of 0 and 30 mM of glucose. **(B)** Changes in the cell morphology of podocytes in the presence of 0 and 9 μM of angiotensin II. **(C)** The migration of podocytes in the presence of 0 and 30 mM of glucose was measured with a transwell assay. **(D)** The migration of podocytes in the presence of 0 and 9 μM of angiotensin II was measured with a transwell assay. **(E)** Invasion by podocytes in the presence of 0 and 30 mM of glucose was measured with a Matrigel assay. **(F)** Invasion by podocytes in the presence of 0 and 9 μM of angiotensin II was measured with a Matrigel assay.

suppressed using antisense RNA. Three TCF8 antisense RNA sequences were transfected into podocytes. As shown in Figures 4C and 4D, TCF8 expression was down-regulated according to Western blotting and qRT-PCR.

3.4. *Silencing TCF8 reversed EMT that was induced with a high concentration of glucose or angiotensin II*

As shown in Figures 5A and 5B, the expression of the epithelial marker molecules E-cadherin and α-catenin

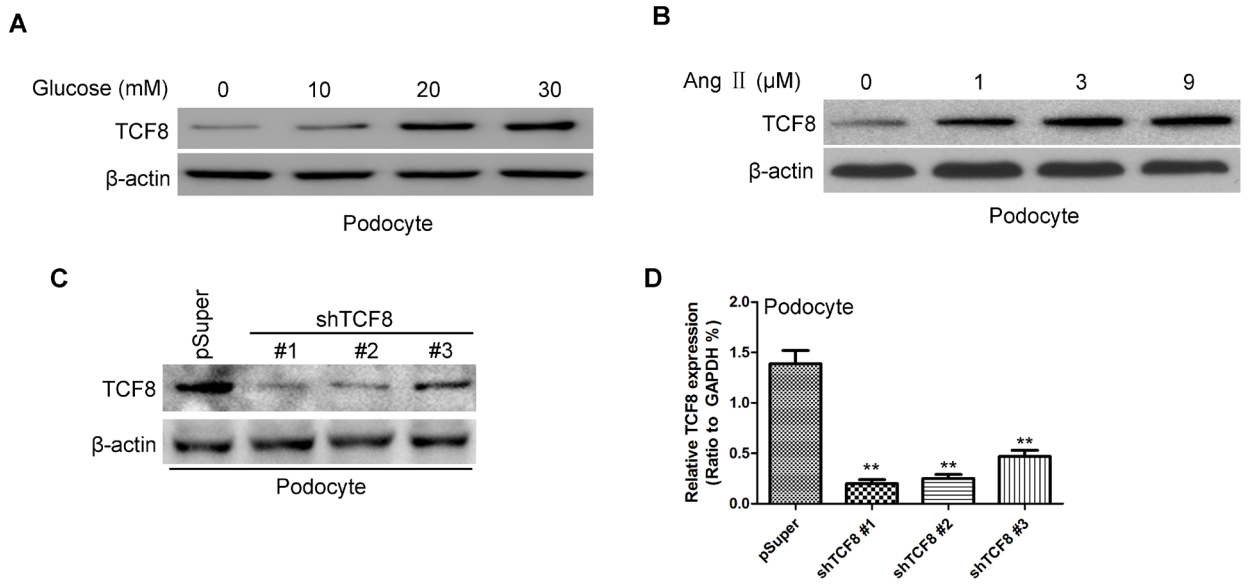

Figure 4. A high concentration of glucose or angiotensin II promoted the expression of TCF8 in podocytes. (A) Podocytes were incubated with 0-30 mM of glucose for 24 h and TCF8 expression was measured with Western blotting. **(B)** Podocytes were incubated with 0-9 μM of angiotensin II for 24 h and TCF8 expression was measured with Western blotting. **(C)** Expression of TCF8 protein after three TCF8 antisense RNA sequences were transfected into podocytes was assayed using Western blotting. **(D)** Expression of TCF8 mRNA after three TCF8 antisense RNA sequences were transfected into podocytes was assayed using qRT-PCR.

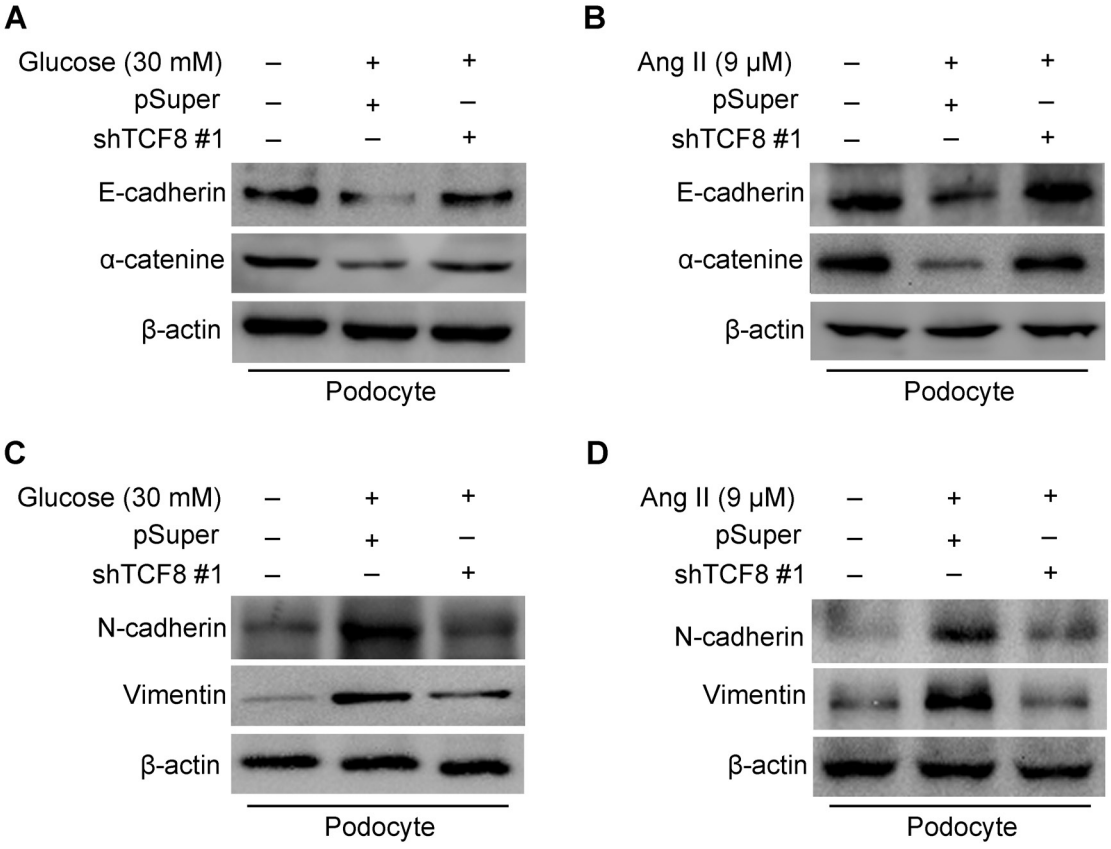

Figure 5. Silencing TCF8 expression in podocytes reversed expression of EMT biomarkers induced with glucose and angiotensin II. (A) Podocytes with silenced TCF8 were incubated with 0 or 30 mM of glucose for 24 h and expression of the epithelial biomarkers E-cadherin and α-catenin was measured with Western blotting. **(B)** Podocytes with silenced TCF8 were incubated with 0 or 9 μM of angiotensin II for 24 h and expression of the epithelial biomarkers E-cadherin and α-catenin expression was measured with Western blotting. **(C)** Podocytes with silenced TCF8 were incubated with 0 or 30 mM of glucose for 24 h and expression of the mesenchymal biomarkers N-cadherin and vimentin was measured with Western blotting. **(D)** Podocytes with silenced TCF8 were incubated with 0 or 9 μM of angiotensin II for 24 h and expression of the mesenchymal biomarkers N-cadherin and vimentin was measured with Western blotting.

decreased with an increase in the glucose or angiotensin II concentration, but this phenomenon was reversed in podocytes with silenced TCF8. As shown in Figures 5C and 5D, the expression of the mesenchymal marker molecules N-cadherin and vimentin increased with an increase in the glucose or angiotensin II concentration,

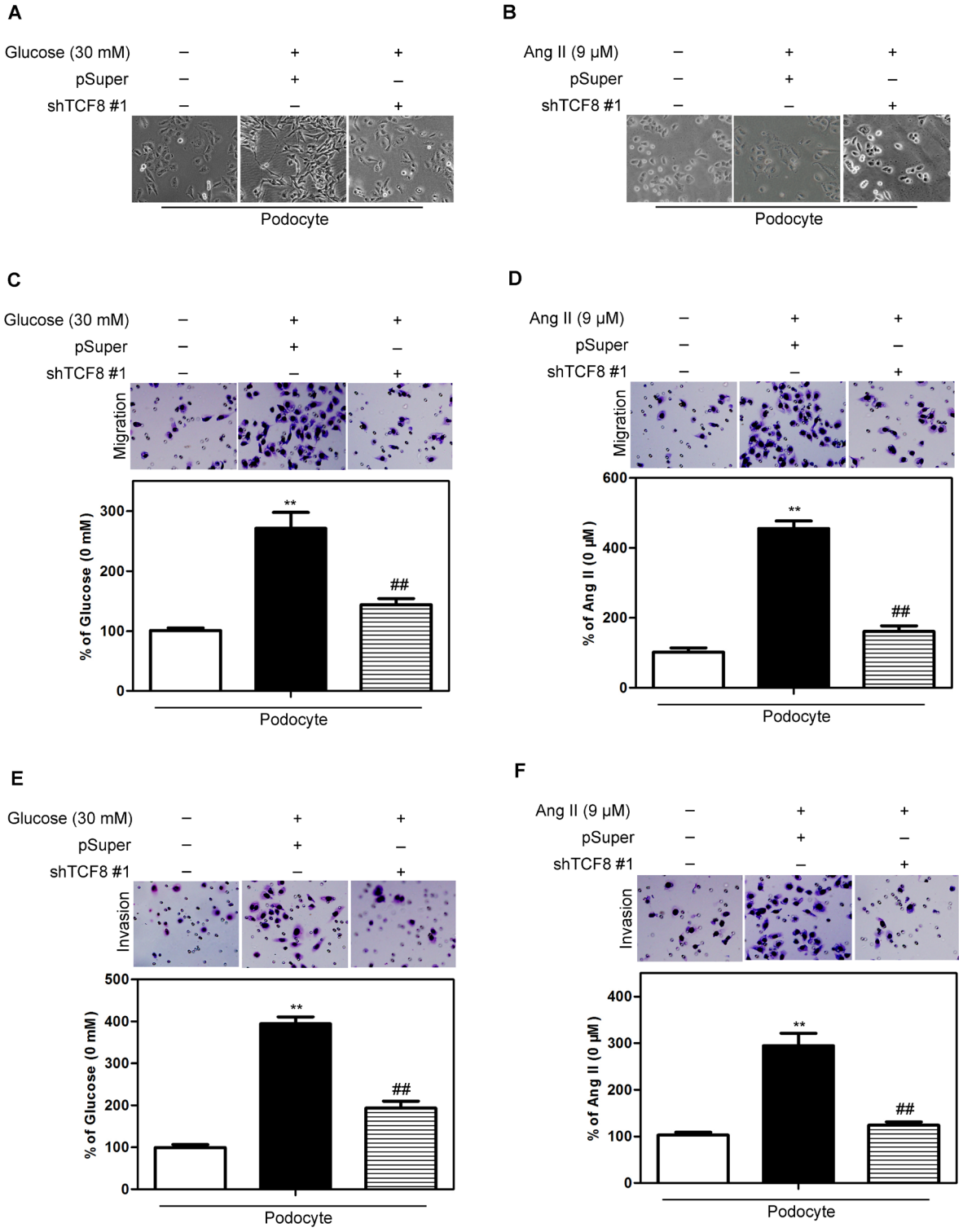

Figure 6. Silencing TCF8 expression reversed changes in the cell morphology of podocytes and podocyte migration and invasion induced with a high concentration of glucose or angiotensin II. (A) Normal podocytes and changes in in the cell morphology of podocytes with silenced TCF8 in the presence of 0 and 30 mM of glucose. **(B)** Normal podocytes and changes in the cell morphology of podocytes with silenced TCF8 in the presence of 0 and 9 μM of angiotensin II. **(C)** Migration of normal podocytes and podocytes with silenced TCF8 in the presence of 0 and 30 mM of glucose. **(D)** Migration of normal podocytes and podocytes with silenced TCF8 in the presence of 0 and 9 μM of angiotensin II. **(E)** Invasion by normal podocytes and podocytes with silenced TCF8 in the presence of 0 and 30 mM of glucose. **(F)** Invasion by normal podocytes and podocytes with silenced TCF8 in the presence of 0 and 9 μM of angiotensin II.

but this phenomenon was reversed in podocytes with silenced TCF8. Results indicated that silencing TCF8 reversed EMT that was induced with a high concentration of glucose or angiotensin II.

3.5. *Silencing TCF8 reversed changes in podocyte morphology and podocyte migration and invasion induced with a high concentration of glucose or angiotensin II*

As shown in Figures 6A and 6B, morphological changes induced with a high concentration of glucose or angiotensin II were reversed in podocytes with silenced TCF8. As shown in Figures 6C and 6D, a higher rate of cell migration was induced with a high concentration of glucose or angiotensin II, but this phenomenon was reversed in podocytes with silenced TCF8. As shown in Figures 6E and 6F, a higher rate of cell invasion was induced with a high concentration of glucose or angiotensin II, but this phenomenon was reversed in podocytes with silenced TCF8. In conclusion, silencing TCF8 reversed changes in podocyte morphology and podocyte migration and invasion induced with a high concentration of glucose or angiotensin II.

4. Discussion

Podocyte injury is a key factor for proteinuria in some kidney diseases (*2*). Proteinuria was assumed to be the result of podocyte depletion due to apoptosis (*2*). However, a recent study has indicated that the podocyte count did not change significantly when microalbuminuria was evident in diabetic rats (*18*). In addition, podocyte detachment and apoptosis lag far behind the onset of proteinuria. Whether initial podocyte injury leads to the onset of proteinuria is uncertain (*3*). Emerging evidence has indicated that podocytes can undergo EMT when exposed to specific pathological conditions, and this may explain the onset of proteinuria. A study has described the phenotypic and morphological changes in diseased podocytes as an EMT (*19*).

Mounting evidence has revealed that glucose and angiotensin II are two key mediators associated with progressive renal injury in chronic kidney disease, and specifically in hypertensive and diabetic nephropathy (in which progressive renal fibrosis is a major determinant of clinical outcomes) (*16*). The current study characterized the role of TCF8 in EMT in podocytes. EMT markers were detected in the presence of a high concentration of glucose or angiotensin II. Results indicated that a high concentration of glucose or angiotensin II promoted EMT in podocytes, and results also indicated that a high concentration of glucose and angiotensin II caused changes in podocyte morphology and they promoted podocyte migration and invasion. Moreover, results indicated that TCF8 is significantly overexpressed in the presence of a high concentration of glucose or angiotensin II. Silencing TCF8 reversed EMT in podocytes and changes in podocyte morphology, and it also reversed the increase in podocyte migration and invasion. Collectively, these findings are the first to suggest that TCF8 is involved in EMT in podocytes.

Recent studies have indicated that EMTs are not limited to renal epithelial cells but that EMTs also occur in endothelial cells and podocytes (*20*). EMT in podocytes may be reversible before podocytes decrease and die off. Podocytes may have biological characteristics causing an EMT under pathological conditions, and an EMT could conceivably result. Podocytes may undergo some changes such as cell detachment, apoptosis, and an EMT when subjected to a harmful stimulus, and the severity and duration of those changes may depend on the specific injury. However, there are numerous unknowns regarding the mechanism of EMT in podocytes and how an EMT in podocytes leads to glomerular disease.

TCF8, also known as ZEB1 or δ EF1, is located on the short arm of human chromosome 10 (*21*). TCF8 is characterized by a homeodomain flanked by two zinc finger domains, and TCF8 encodes 1108 amino acids. An important molecular event in EMT is the downregulation of E-cadherin. TCF8 can bind with E-cadherin promoter and inhibit the expression of E-cadherin. TCF8 has been found to be closely related to EMT in tumors, but its role in EMT in podocytes has yet to be elucidated.

In conclusion, the current results revealed that TCF8 was generally overexpressed in podocytes in the presence of a high concentration of glucose or angiotensin II and that TCF8 was involved in EMT. These results indicate that TCF8 plays a key role in promoted EMT in podocytes and that TCF8 may be an effective novel therapeutic target for the management of glomerular disease.

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(Received November 29, 2016; Revised December 27, 2016; Accepted December 31, 2016)