SnoN as a Key Regulator of the High Glucose-Induced Epithelial-Mesenchymal Transition in Cells of the Proximal Tubule

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Key Words

Ski-related protein N · Proximal tubular epithelial cells · Epithelial-mesenchymal transition · Transforming growth factor- β 1

Abstract

Background/Aims: Ski-related protein N (SnoN) suppression is essential to transforming growth factor- β 1 induction and the epithelial-mesenchymal transition (EMT) in several cancer cells. The role of SnoN in diabetic nephropathy is unknown. We aimed to determine the role of SnoN in the EMT of proximal tubule cells (PTCs) maintained under high glucose conditions. **Methods:** Immunohistochemistry, immunocytochemistry, Western blotting, small interfering RNA gene silencing, viral transduction and RT-PCR were used to assess changes in SnoN, E-cadherin, cytokeratin-18, α -smooth muscle actin and fibronectin expression using an in vivo streptozotocin-induced rat diabetic nephropathy model, and PTCs exposed to high glucose (25 mmol/l). **Results:** High glucose induced EMT in vitro and in vivo. Exposure of PTCs to a high concentration of glucose suppressed SnoN expression in a time-dependent manner compared with normal glucose and high osmolarity-treated groups. SnoN gene silencing under high glucose conditions appears to enhance the transition of PTC phenotype. Conversely, ectopic

expression of exogenous SnoN after transfection conferred tubular epithelial cell resistance to high glucose-induced EMT. **Conclusion:** SnoN plays a negative role in high glucose-induced EMT in PTCs. The effect of SnoN downregulation in vivo and in vitro suggests that SnoN may be a potential therapeutic target.

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Introduction

Renal fibrosis is characterized by distinctive histopathological features including glomerulosclerosis, interstitial fibrosis, and tubular atrophy [1], whereby resident renal fibroblasts are traditionally considered to be the principal mediators of fibrosis. However, evidence now exists to suggest that the appearance of interstitial myofibroblasts is also critical, with myofibroblasts being more profibrotic than resident interstitial fibroblasts. Moreover, the proximal tubule cells (PTCs) also play an important role in the deposition of the extracellular matrix (ECM). Similar to myofibroblasts, PTCs are of mesenchymal origin and appear capable of reverting to a mesenchymal phenotype via a process known as the epithelial-mesenchymal transition (EMT). This occurs in response to certain physiological cues [2, 3]. Defined as a

process whereby PTCs lose their epithelial phenotype and acquire new mesenchymal characteristics, EMT is tightly regulated and largely driven by the pleiotropic cytokine transforming growth factor- β 1 (TGF- β 1) [4]. It has been suggested that EMT contributes to up to one third of the total interstitial fibroblast population [5, 6], with disruption of the tubular basement membrane being a necessary step in facilitating this transition [7].

Previous findings from this laboratory have shown that TGF- β 1 plays an important role in the renal fibrosis of diabetic nephropathy (DN) [8]. The Ski-related protein N (SnoN) has been identified as a Smad transcriptional co-repressor, and is known to form part of the regulatory component within the nuclei that occurs during the final stage of TGF- β 1/Smad signaling [9, 10]. The abundance and activity of SnoN in a particular cellular context may determine the ultimate response of cells to TGF- β 1 stimulation.

Recently, Yang et al. [11] reported that there is a progressive downregulation of the SnoN protein in the fibrotic kidney after unilateral ureteral obstruction. Their work also indicated that the loss of SnoN has a profound impact on Smad signaling, based on the finding that SnoN knockdown via small interfering RNA (siRNA), dramatically sensitizes PTCs to TGF- β 1 stimulation. Moreover, increased SnoN expression, either via transfection of a SnoN expression vector or through hepatocyte growth factor induction [12], led to the suppression of TGF- β 1/Smad-mediated gene transcription. However, whether SnoN has similar effects in PTCs under high glucose conditions remains unclear. The aim of the present study was to establish the nature of total SnoN expression in the PTCs both in vivo and in vitro, and to examine the role of SnoN in the EMT mediated by exposure to high glucose concentrations.

Materials and Methods

Animals

All animal experiments were conducted in accordance with guidelines of the National Health and Medical Research Council of China's Code for the Care and Use of Animals for Scientific Purpose. Male SD rats (Slack Animal Co., China) weighing 180 ± 200 g were randomized to receive either 60 mg/kg of streptozotocin (STZ; Sigma, USA) diluted in 0.1 mol/l citrate buffer pH = 4.5 or citrate buffer (nondiabetic) by tail vein injection. The rats were monitored weekly for changes in weight and blood glucose levels, and only STZ-treated animals with blood glucose ≥ 16.7 mmol/l were considered diabetic. All animals were housed in a stable environment maintained at $20 \pm 1^\circ\text{C}$ with a 12:12-hour light-dark cycle commencing at 6 a.m. Diabetic and age-matched nondiabetic rats ($n = 6$) were killed 2, 4, 8 and 12 weeks

after the STZ injection. Both kidneys were removed and weighed, with one being fixed in 4% paraformaldehyde; the other kidney was snap-frozen in liquid nitrogen frozen and then stored at -80°C for subsequent use.

Isolation and Culture of Primary PTCs

The rat primary PTCs were harvested, dissociated and digested as described previously with modifications [13, 14]. Briefly, the kidneys were removed aseptically, the medulla was dissected out and the cortex fragmented, and forced through a graded series of stainless steel sieves (80- and 100- μm mesh). The proximal tubular fragment was then carefully removed from the surface of the secondary sieve, suspended in normal saline and centrifuged at 1,500 g for 10 min at 4°C . The pellet was then resuspended in Dulbecco's modified Eagle medium (DMEM; Hyclone, USA) supplemented with insulin (0.4 $\mu\text{l/ml}$; Becton Dickinson, China), human transferrin (17.5 $\mu\text{g/ml}$, Sigma, USA) and recentrifuged. The resulting pellet was resuspended in pre-warmed phosphate-buffered solution (PBS) containing of 0.25% trypsin, and incubated with shaking at 37°C for 30 min. The cells were subcultured in 25 cm^2 culture flasks. Passages 3 were characterized and used in subsequent expressions.

Cell Treatments

Once the cultures had reached 80–90% confluence, the cells were rendered quiescent by incubation in fresh serum-free DMEM for 24 h. After 24 h, the cells were incubated in 2% fetal bovine serum (Invitrogen, USA) containing different concentrations of glucose, defined as: normal glucose (5.5 mmol/l), high glucose (5.5 mmol/l glucose + 19.5 mmol/l D-glucose) or high osmolarity (5.5 mmol/l glucose + 19.5 mmol/l D-mannitol). The cells were incubated for 30 min, 2, 12, 24, 48, 72 and 96 h. Whole-cell lysates were then collected and stored at -80°C for Western blot analysis.

Biochemical Analysis

Each week, the rats were weighed, and their blood glucose levels were measured. At the end of each 2, 4, 8 or 12 week experimentation period, the rats were housed in individual metabolic cages in order collect urine over a 24-hour period for the measurement of protein in the urine (as determined by the Bradford method). In parallel, whole blood was collected from the anesthetized rats via femoral artery. The blood was centrifuged at 3,000 g for 15 min at 4°C to obtain serum for measuring creatinine (Scr) levels.

Histopathological Analysis

The kidneys were fixed in paraformaldehyde and embedded in paraffin. Transverse sections (4 μm) were then cut, and Masson's trichrome and hematoxylin-eosin (HE) staining were then performed.

Gene Silencing by siRNA

The generation of sequence-specific siRNA duplexes was carried out using a silencer SnoN siRNA construction kit essentially according to the manufacturer's instructions (Santa Cruz, Calif., USA). The SnoN and control siRNA (fluorescein conjugates) were designed and chemically synthesized by Santa Cruz. The cells were cultured in complete medium for 24 h prior to transfection (2 ml media in each well of 6-well plate) at which time the medium was changed to serum and antibiotic-free medium. Cells were then incubated at 37°C until 60–80% confluent. The SnoN siRNA and control siRNA (20 $\mu\text{mol/l}$) were diluted into the 100 μl siRNA

transfection medium, and the siRNA duplex solution was added directly to the diluted transfection reagent and incubated for 30 min at room temperature. The mixture was then added to the cells and incubated for 6 h in CO₂ incubator, and 1 ml of normal growth medium containing twice the normal serum and antibiotic concentration (2× normal growth medium) was added without removing the transfection mixture. The cells were incubated in a medium containing normal and high glucose concentrations prior to RNA and protein isolation.

Cell Culture and Viral Transduction

HK-2 cells were grown and maintained in the recommended medium, DMEM/Ham's F12 (50/50) supplemented with 10% fetal bovine serum, L-glutamine, insulin, transferrin, sodium selenite, epidermal growth factor (2.5 ng/ml), and pituitary extract (1.5 µg/ml) in a humidified atmosphere of 5% CO₂. Human SnoN cDNA was transferred from pCI-Neo HA-SnoN vector into the retroviral pBMN-I-GFP vector (both from Addgene). The phoenix Eco cells were transfected with SnoN retroviral vector (20 µg of plasmid DNA/100-mm plate) using the calcium phosphate precipitation method. Viral supernatants were harvested 48 h later, supplemented with 5 µg/ml of Polybrene, and used without further dilution for the infection of the HK-2. After 24-hour incubation, they were incubated for a further 24 h with or without a high concentration of glucose.

Immunohistochemistry

Briefly, 3-µm sections were placed into histosol to remove the paraffin, rehydrated through graded ethanol, and incubated for 30 min with normal rat serum. The sections were then incubated with anti-SnoN (1:100, Santa Cruz, USA), anti-E-cadherin (1:50, Santa Cruz), anti-α-smooth muscle actin (α-SMA, 1:100, Santa Cruz) and fibronectin (FN, 1:50, Santa Cruz) antibodies overnight (18 h) at 4°C. The following day, the sections were incubated with a biotinylated swine anti-rabbit IgG antibody (1:200, DAKO, Denmark), followed by treatment with an avidin-biotin peroxidase complex (1:200). Localization of the peroxidase conjugates was achieved by using diaminobenzidine tetrahydrochloride as a chromagen. The sections were counterstained in Mayer's hematoxylin. The sections incubated in normal rabbit serum (1:10) served as the negative controls.

Immunocytochemistry

The cells were grown on 6-well plates. Once the cultures had reached 50–70% confluence, the cells were washed twice with cold PBS, fixed on ice in a 1:1 methanol:acetone solution for 10 min, and air dried for 2 min. After blocking with 20% normal rabbit serum diluted in PBS for 30 min, the cells were incubated with anti-α-SMA (1:100), anti-cytokeratin-18 (CK-18, 1:100, Santa Cruz) or SnoN (1:100) antibodies overnight at 4°C. The following day, the sections were incubated with a biotinylated swine anti-rabbit IgG antibody (1:200), followed by an avidin-biotin peroxidase complex (1:200). To visualize the localization of peroxidase conjugates, the cells were stained with diaminobenzidine tetrahydrochloride. The cells were then viewed with a LEICA microscope.

Western Blotting

Protein expression in the kidney tissue and cultured cells was analyzed by Western blotting. The primary antibodies used were as follows: anti-CK-18 (1:200), anti-E-cadherin (1:200), anti-α-

SMA (1:200), anti-FN (1:200), anti-SnoN (1:400) and anti-β-actin (1:500, Abcam, UK). Quantification of the Western blot data was performed by measuring the intensity of the hybridization signals using a Quantity one 4.6 Image analysis program. Protein expression levels were calculated after normalizing with β-actin.

RT-PCR

Total RNA was extracted with Trizol (Invitrogen) in accordance with the manufacturer's recommendations. The RT-PCR was performed using a SuperScript III One-Step RT-PCR System and Platinum Taq DNA polymerase (Invitrogen). The number of amplification cycles in the semi-quantitative PCR was determined from the linear portion of the PCR cycle, and amplification was performed with increasing numbers of cycles for SnoN and β-actin. The SnoN primer was obtained from Santa Cruz. The β-actin primers were as follows: β-actin (306 bp) forward 5'-TGG CAT TGT GAT GGA CTC-3', and reverse 5'-CCG ATA GTG ATG ACC TGA C-3'. The amplified SnoN and β-actin products were separated by electrophoresis on 1% agarose gels and then visualized by ethidium bromide staining. The bands were scanned on a Gel documentation system (Bio-Rad) and quantified by densitometry using Quantity One software, with β-actin used as an internal control for sample normalization.

Statistical Analysis

All data were expressed as mean ± SD. Statistical analysis of the data was performed using SPSS 13.0 software. Comparison among groups was made using one-way ANOVA followed by the Student-Newman-Keuls test. Differences were considered significant when p values were <0.05.

Results

Primary Culture of PTCs

The characteristic cobblestone morphology of epithelia showed that optimal conditions for the primary culture of PTCs were successfully established. Both immunocytochemistry and Western blotting revealed that the cells were positive for CK-18 and E-cadherin, but negative for α-SMA (fig. 1), confirming that the cells were of proximal tubular epithelial origin.

Animal Data

From week 4, the kidney index (defined as the ratio of kidney weight to bodyweight), a marker for the development of DN, was significantly increased. Diabetic rats also exhibited increased Scr, blood glucose and 24-hour urinary protein excretion at all time points (table 1).

Histology

Both HE and Masson's trichrome staining showed a typical histologic phenotype in normal rats (fig. 2a, d). In contrast, for the STZ-treated rats, the kidneys showed increased ECM deposition within the tubulointerstitium and

Fig. 1. Characterization of primary PTCs. **a** Rat PTCs form a tight cobblestone-like monolayer after 3 days in culture. **b** Expression of CK-18 in rat PTCs, compared with **c** a lack of α -SMA expression in the cells. **d** Western blotting demonstrating CK-18, E-cadherin and α -SMA expression. **a** $\times 200$. **b**, **c** $\times 400$.

Fig. 2. Diabetes-induced ECM accumulation and tubulointerstitial fibrosis. The panels show the results of HE (**a–c**) and Masson trichrome (**d–f**) staining in kidney sections from the control animals (**a** and **d**), and those injected with STZ 4 weeks (**b** and **e**) or 12 weeks (**c** and **f**) previously. The animals treated with STZ demonstrated ECM deposition in the cortical sections of the kidney. All images taken at $\times 400$ magnification.

tubular basement membrane thickening, which occurred at 4 and 12 weeks following treatment (fig. 2b, c, e, f).

EMT Markers in Tubular Epithelial Cells under High Glucose Concentrations in vitro and in vivo

Western blot analysis revealed that exposure to high glucose concentrations suppressed E-cadherin and CK-

18 expression (fig. 3), but induced α -SMA and FN expression (fig. 4) in the PTCs. In vivo, at all time points after the induction of diabetes, E-cadherin staining in the tubules of diabetic SD rats was reduced compared with the control animals (fig. 5a–c), while α -SMA and FN expression in the tubular cells at week 12 was increased. The number of tubular cells that stained positive for E-cad-

Table 1. Phenotypic characteristics of the experimental animals, including blood glucose, Scr, 24-hour urine protein excretion, bodyweight, and kidney index

Time	Group	Blood glucose mmol/l	Scr μmol/l	24-hour urine protein, mg	Bodyweight g	Kidney index mg/g
2 weeks	Normal	6.72 ± 2.42	58.86 ± 7.40	2.01 ± 1.21	283.07 ± 38.37	7.11 ± 0.48
	Diabetic	20.42 ± 5.11*	71.37 ± 7.62	22.36 ± 8.91*	285.42 ± 32.08	11.27 ± 0.58
4 weeks	Normal	7.61 ± 0.55	53.21 ± 3.21	5.28 ± 2.31	327.32 ± 40.93	7.23 ± 0.58
	Diabetic	24.07 ± 3.52*	72.55 ± 5.36	32.30 ± 8.35*	283.40 ± 43.09	12.02 ± 0.83*
8 weeks	Normal	7.89 ± 1.34	54.93 ± 7.59	7.73 ± 1.22	340.62 ± 76.19	5.91 ± 0.41
	Diabetic	26.36 ± 2.02*	99.23 ± 12.11*	27.26 ± 8.51*	274.18 ± 12.31*	13.21 ± 1.59*
12 weeks	Normal	7.00 ± 0.71	51.14 ± 10.43	3.41 ± 1.71	397.67 ± 85.08	5.69 ± 0.52
	Diabetic	23.43 ± 2.56*	97.80 ± 21.87*	22.10 ± 7.31*	276.13 ± 13.98*	12.59 ± 2.46*

Values represent mean ± SD (n = 6). * p < 0.05 versus normal groups at the same time points.

herin was 9.83 ± 1.17 in the diabetic rats versus 17.50 ± 1.87 in control rats. No positive α -SMA expression was detected in the tubules of diabetic rats before week 12, or in the control rats at any time point. Staining for α -SMA was 1.02 ± 0.48 in the tubules of the diabetic rats at week 12. The expression of FN was also increased in the diabetic rats, suggesting that the PTCs had begun to lose their epithelial phenotype and commenced expressing myofibroblastic markers.

SnoN Expression in Diabetic Conditions in vitro and in vivo

SnoN expression in both the cells exposed to high glucose concentrations and in kidneys of the STZ-induced diabetic SD rats was measured. Exposure of PTCs to 25 mmol/l D-glucose significantly suppressed SnoN protein expression in a time-dependent manner, compared with normal glucose and high osmolarity-treated cells (fig. 6). As described by Yang et al. [11], SnoN was predominantly localized to the renal tubular epithelium and interstitium of the kidney. While strong staining was observed in the control animals, in the STZ-treated animals the number of SnoN-positive tubules decreased from week 4. Western blot analysis also demonstrated a time-dependent decrease in the expression of SnoN protein in the diabetic rats (fig. 7).

EMT Markers in SnoN-Silenced PTCs

SnoN siRNA transfection in the PTCs resulted in markedly reduced SnoN mRNA and protein expression at 24 h (fig. 8). E-cadherin and CK-18 expression were re-

duced by 54.61 and 65.45%, respectively, compared with the control levels in the group exposed to high glucose concentrations (fig. 9). In comparison, both α -SMA and FN expression increased when compared with the high glucose group, but also decreased relative to the normal glucose group (fig. 9). Moreover, there was no significant difference in CK-18, E-cadherin and FN expression in SnoN siRNA-transfected cells compared with control levels under normal glucose concentrations (fig. 10). Similarly, α -SMA abundance was also unaltered after SnoN knockdown (data not show).

Expression of EMT Markers following the Overexpression of SnoN PTCs

To conform further the important role of SnoN in high glucose-induced EMT of PTCs, we studied the effect of SnoN overexpression on EMT marker levels in cells exposed to high glucose concentrations. The HK-2 cells were transduced with SnoN or control retroviruses, and 24 h later they were incubated for 24 h with or without high glucose concentration. As shown in figure 11, overexpression of SnoN led to a reduction of α -SMA and FN expression by 42.78 and 50.11%, respectively, compared with the control levels in the group exposed to the high glucose concentrations. This effect was statistically significant. Both E-cadherin and CK-18 expression increased compared with the high glucose group, but decreased relative to the normal glucose group (fig. 11). The lack of a stronger inhibition of the EMT markers by SnoN may be due to the low efficiency of viral transduction, as SnoN only increased by 50.26% (fig. 12).

Fig. 3. Exposure to high glucose concentrations suppressed CK-18 and E-cadherin expression in the PTCs. **a** Western blot analysis showed a time-dependent decrease in CK-18 and E-cadherin expression. **b, c** Relative abundance of CK-18 (**b**) and E-cadherin (**c**) in the different groups after β -actin normalization. Data are presented as mean $\pm$ SD of four separate experiments. * $p < 0.05$ versus control, # $p < 0.05$ versus mannitol group at the equivalent time point.

Fig. 4. Exposure to high glucose-induced α -SMA and FN expression in the PTCs. **a** Western blot analysis showed a time-dependent increase in α -SMA and FN expression. **b, c** Relative abundance of α -SMA (**b**) and FN (**c**) in the different groups after β -actin normalization. Data are presented as mean $\pm$ SD of four separate experiments. * $p < 0.05$ versus control, # $p < 0.05$ versus mannitol group at the equivalent time point.

Fig. 5. Markers of EMT in the STZ-induced diabetic rats. **a, b** Representative photographs of E-cadherin immunohistochemistry in the normal (**a**) and diabetic rats (**b**) at 12 weeks (arrowhead). **d, e** α -SMA was detected in the tubules of the diabetic rats (**d**) at 12 weeks (arrowhead), but in the normal rats there was no expression of α -SMA in the tubules (**e**). **g, h** Representative images of FN immunohistochemistry in the normal (**g**) and diabetic rats (**h**) at 12 weeks (arrowhead). **a, b, d, e, g, h** $\times 400$. **c, f, i** Quantification

of E-cadherin, α -SMA and FN expression in the normal and diabetic rats. Compared with the control animals, there was a reduction in E-cadherin (**c**) expression but an increase in α -SMA (**f**) and FN (**i**) expression in the proximal tubules of the diabetic rats. The data reflect the average number of renal tubules from 10 separate images of the kidney cortex showing positive staining. $\times 400$. Data are expressed as mean $\pm$ SD, * $p < 0.05$ versus normal.

Discussion

The findings in this study confirm that the expression of the transcription factor SnoN in tubule cells is decreased under diabetic conditions in vivo and following exposure to high glucose concentrations in vitro. High glucose concentrations [15, 16] are known to increase intracellular TGF- β 1 expression in the kidney and contrib-

ute to the development and progression of interstitial fibrosis through mechanisms including EMT [17, 18]. Moreover, TGF- β 1 has long been recognized as a pivotal driver of EMT both in vivo and in vitro [19, 20]. The diabetic SD rat is a model that develops progressive renal injury with tubulointerstitial fibrosis. In this study, we demonstrated decreased expression of total SnoN in the tubules, suggesting that downregulation of SnoN is a

Fig. 8. The effect of SnoN siRNA-mediated gene silencing. The SnoN siRNA was transfected into PTCs cultured in 6-well plates. In parallel, control cells were transfected with nonspecific siRNA (control). **a** Western blot data showing the expression of SnoN protein in the PTCs transfected with SnoN siRNA. Treatment with siRNA for SnoN led to a 48.63% reduction in SnoN expression. **b** RT-PCR showed that SnoN siRNA transfection led to a 54.41% reduction in SnoN mRNA expression. Data are presented as the mean $\pm$ SD of three separate experiments. * $p < 0.05$ versus control.

Fig. 9. The effect of SnoN knockdown on high glucose-induced EMT in PTCs. The cultured PTCs were treated with SnoN siRNA, then with 25 mmol/l glucose for 24 h. The expression of CK-18, E-cadherin, α -SMA and FN protein was assessed by Western blot analysis. **a-d** CK-18 (**a**), E-cadherin (**b**), α -SMA (**c**) and FN (**d**) expression in SnoN knockdown cells. Data are presented as the mean $\pm$ SD of three separate experiments. * $p < 0.05$ versus normal glucose, # $p < 0.05$ versus high glucose.

Fig. 10. The effect of SnoN knockdown on CK-18, E-cadherin and FN expression in PTCs under normal glucose concentrations. The cultured PTCs were treated with a SnoN siRNA, then with normal glucose for 24 h. The expression of CK-18, E-cadherin and FN protein was assessed by Western blot analysis.

Fig. 12. The effect of SnoN retroviruses on SnoN protein expression in HK-2 cells. Western blotting showing the expression of SnoN protein in HK-2 transfected with SnoN retroviruses. Treatment with SnoN retroviruses led to a 50.26% increase in SnoN protein. Data are presented as mean $\pm$ SD of three separate experiments. * $p < 0.05$ versus control.

Fig. 11. The effect of SnoN overexpression on high glucose-induced EMT in HK-2. The HK-2 cells were transduced with SnoN or control retroviruses, then with 25 mmol/l glucose and normal glucose for 24 h. The expression of CK-18, E-cadherin, α-SMA and FN protein was assessed by Western blot analysis. **a-d** CK-18

(**a**), E-cadherin (**b**), α-SMA (**c**) and FN (**d**) expression in SnoN overexpression cells. Data are presented as mean $\pm$ SD of three separate experiments. * $p < 0.05$ versus normal glucose, # $p < 0.05$ versus high glucose.

precursor to EMT occurring in PTCs. Furthermore, we also demonstrated that SnoN silencing promotes the development of EMT in PTCs exposed to high glucose concentrations, through decreased CK-18 and E-cadherin expression, and increased α -SMA and FN expression, and that overexpression of SnoN partially inhibits the induction of EMT by high glucose concentrations. Although this might suggest that the PTCs used were not fully 'epithelial' to begin with, we ensured minimal heterogeneity by using primary cultured cells, which constitute the major type of renal epithelial cells, and only used early-passage cells for our experiments. As it is acknowledged that primary cultured cells in the same culture show marked heterogeneity with respect to epithelial characteristics, the ability of SnoN silencing to reduce CK-18 and E-cadherin expression (and increase α -SMA and FN expression) is noteworthy and affirms SnoN's role in EMT.

SnoN is a transcriptional corepressor of the TGF- β 1 promoter [10], and Yang et al. [11] previously demonstrated a temporal link between unilateral ureteral obstruction, SnoN and TGF- β 1 in the induction of renal fibrosis. The similarities with diabetes-induced renal fibrosis, in particular DN, where the generation of TGF- β 1 is a key pathogenic feature, are striking. Our data revealed SnoN expression was significantly decreased in the tubules of the diabetic rat at 4 weeks. At the same time, the PTCs of these animals were demonstrating early phenotypic features of EMT: a reduction in CK-18 and E-cadherin expression and an increase in the expression of the FN. Furthermore, *in vitro* exposure to high glucose concentrations led to a time-dependent reduction in SnoN expression in primary cultured PTCs. The expression of both CK-18 and E-cadherin was decreased, whereas α -SMA and FN expression was increased in PTCs following SnoN silencing. Although the molecular mechanisms responsible for the suppression of SnoN protein expression following exposure to high glucose concentrations are unknown, increased osmolarity owing to high glucose may be one of the contributing factors. However, the significant difference between the mannitol and glucose groups (in which osmolarity was comparable) suggested that a specific effect of glucose unrelated to corresponding hyperosmolality should be considered. Together, these data intimate that total SnoN expression is decreased under conditions known to cause EMT, and its knockdown promotes the development of EMT in PTCs exposed to high glucose.

The fact that SnoN was downregulated under conditions known to cause EMT is not surprising given its

known role as a TGF- β 1 negative regulator. In this context, SnoN acts by interacting directly with Smad2, Smad3, and Smad4, and represses their ability to activate the expression of TGF- β 1 target genes. Furthermore, TGF- β 1 has been shown to promote tumor EMT through both Smad-dependent and Smad-independent pathways [21–24]. Because SnoN is a negative regulator of the Smad proteins, one would predict that SnoN may operate in the opposite direction as TGF- β 1 during tumorigenesis particularly if these activities are dependent upon its ability to bind to the Smad proteins. Within the setting of cancer, the role of the SnoN-antagonistic TGF- β 1-induced EMT has been defined in several epithelial cell cancer lines [22, 25–27]. Several mechanisms have been proposed to explain how SnoN blocks Smad-mediated gene transcription. It has been reported that SnoN can sequester Smad proteins in the cytoplasm, blocking Smad translocation to the nucleus [28, 29]. In addition, SnoN can physically bind to, and interact with, activated Smad in the nuclei. By doing so, the transactivation capacity of Smad is sequestered by co-repressors [9, 10]. Irrespective of the mechanism, our results suggested that SnoN also has a similar role in the pathological process of high glucose-induced tubular EMT.

In summary, the findings presented here demonstrate for the first time that SnoN expression is decreased under conditions known to promote kidney injury and fibrosis, specifically EMT, in an animal model of progressive DN. In addition, the knockdown of SnoN promoted EMT in primary cultured PTCs. Given the central role that EMT plays in the development and progression of interstitial fibrosis, the identification of SnoN as a key regulator of high glucose-induced EMT represents an important finding. Further studies may confirm it as a potentially useful target for therapeutic intervention in an attempt to limit EMT and with it the decline in renal function seen in patients with DN.

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