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Snail, a transcriptional regulator, represses nephrin expression in glomerular epithelial cells of nephrotic rats

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Snail is a DNA-binding molecule that plays a pivotal role in regulating cell adhesion and epithelial to mesenchymal transition. Visceral epithelial cells (podocytes) in kidney glomeruli form a sophisticated cell–cell junction called a slit diaphragm that prevents the loss of plasma protein during ultrafiltration. Nephrin, located in the slit diaphragm and critical for maintaining the integrity of this structure, belongs to the class of cell adhesion molecules of the immunoglobulin super-family. As previously reported, the transcriptional activity of nephrin is a determinant of the integrity of the slit diaphragm in puromycin aminonucleoside (PAN) nephrosis rats. Here, we examined the role of Snail in nephrin expression. In accordance with the downregulation of nephrin in PAN nephrosis rats, Snail was upregulated *in vivo* and its DNA-binding activity was stimulated in injured podocytes while normal podocytes did not express Snail. An *in vitro* study demonstrated that Snail bound to E-box motifs in a specific segment of the rat nephrin gene repressed the transcription of nephrin and downregulated nephrin protein. We also found that the expression level of Snail in injured podocytes was regulated by GSK3, which is known to phosphorylate Snail and induce its proteolysis. Pharmacological *in vitro* and *in vivo* inhibition studies of GSK3 suggested that GSK3 activity decreased in injured podocytes and this change partially contributed to the decrease in nephrin and increase in Snail and proteinuria. Concordantly, we found that Wnt-2 was upregulated in injured podocytes and activated the Wnt canonical pathway. As the Wnt canonical pathway inactivates GSK3, it is likely that Wnt-2 accounts for the accumulation of Snail in injured podocytes. In conclusion, Snail is a key molecule, which perturbs the integrity of the slit diaphragm through transcriptional repression of nephrin under pathological conditions. Wnt-GSK3 pathway participates in this mechanism.

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Visceral epithelial cells of renal glomeruli, termed podocytes, form highly specialized epithelium in kidney. In normal kidney, loss of plasma protein during ultrafiltration is prevented by the slit diaphragm, which is a unique cell–cell junction between adjacent podocytes.[1] Recent studies have revealed that injury to podocytes leads to tubular damage and interstitial fibrosis through perturbation of slit diaphragm and resultant proteinuria while severe podocyte injury is an initial step leading to glomerular sclerosis, which is the end stage result of glomerular injury.[2]

The slit diaphragm is comprised of many molecules, such as nephrin, neph1 and members of the cadherin super-family such as P-cadherin and FAT1.[3] Among these molecules, human nephrin gene product (NPHS1) is a key constituent which belongs to a cell adhesion molecule class of the immunoglobulin super-family.[4] Humans with nonsense mutations in NPHS1[5,6] and mice lacking nephrin[7] manifest podocyte injury with massive proteinuria and effacement of podocyte foot processes. Proteinuric diseases, such as minimal change nephropathy, focal segmental glomerulosclerosis, lupus nephritis, and diabetic nephropathy in human and experimental nephropathy in animals show a perturbation of slit diaphragm and a decrease in the expression level of nephrin.[8–18] Although null or decreased expression of nephrin is closely related to podocyte dysfunction and proteinuria, little is known about the transcriptional regulation of nephrin.

We previously reported that all-*trans* retinoic acid drives the *in vitro* transcription of nephrin through retinoid receptors (RARs) which bind to the promoter region of the nephrin gene as well as exerting anti-proteinuric effects in puromycin aminonucleoside (PAN) nephrosis rats and that

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vitamin A-deficiency lead to a persistent proteinuria.[18] Nephrin expression is also under the control of some other signaling pathways in addition to it's regulation by retinoic acids. Wanger *et al*[19] found Wilms' tumor suppressor (WT-1) transcriptionally activates nephrin. Doublier *et al*[20] and Langham *et al*[21] reported that glycated albumin and angiotensin II repress nephrin in diabetic patients and that an angiotensin converting enzyme inhibitor prevents nephrin repression. Recently, Benigni *et al*[22] demonstrated enhanced nephrin transcription through peroxisome proliferator-activated receptor gamma (PPARγ) by pioglitazone, a PPARγ agonist.

This study focuses on the transcriptional regulation of nephrin through the transcriptional regulator Snail, which was originally identified in *Drosophila melanogaster* as a transcriptional repressor of shotgun, an orthologue of E-cadherin.[23] Snail represses claudin, occludin[24,25] and MUC1[26] as well as E-cadherin. Transcriptional repression of these molecules occurs through the consensus DNA-binding site for Snail and this site has been reported as the E-box motif (5′-CA(C/G)(C/G)TG-3′).[27] Snail plays a central role in regulating epithelial functions and is critical for triggering epithelial to mesenchymal transition (EMT) in embryonic development, carcinogenesis, and cancer metastasis.[28]

In this study, we examined the involvement of Snail in the transcriptional regulation of nephrin using a rat nephrosis model.

MATERIALS AND METHODS

Animals

Six-week-old male Sprague–Dawley rats were purchased from Japan SLC Inc. (Hamamatsu, Japan), and were maintained at the animal facility of Osaka University School of Medicine. The PAN nephrosis model for podocyte injury was induced by a single intravenous injection of PAN (Sigma, St Louis, MO, USA). PAN was dissolved in saline and administered at a dose of 10 mg/100 g of body weight (wt). *In vivo* pharmacological inhibition of glycogen synthase kinase 3 (GSK3) was accomplished using 2′Z, 3′E-6-bromoindirubin-3′-oxime (BIO), a cell-permeable specific GSK3 inhibitor (Calbiochem, CA, USA) or vehicle (DMSO) and these were administered intraperitoneally at a dose of 5 μmol/kg body wt/day once a day from the day of PAN injection to the day of killing. At periodic intervals, rats were anesthetized by intraperitoneal administration of pentobarbital and processed as described previously.[18] PAN nephrosis is a highly reproducible proteinuric model. On day 5, all the rats showed marked proteinuria (on the average 61.9 ± 7.69 mg/mg creatinine) while normal rats showed a trace amount (on the average 1.27 ± 0.412 mg/mg creatinine).

Protein Extraction and RNA Extraction

Isolated glomeruli were lysed in the cell lysis buffer (20 mM Tris · HCl (pH 7.5)/150 mM. NaCl/1 mM EDTA/1 mM EGTA/1% Triton/2.5 mM sodium pyrophosphate/1 mM β-glycerophosphate/1 mM Na_3VO_4/leupeptin (1 μg/ml)) (Cell Signaling Technology, Danvers, MA, USA) using a glass/Teflon homogenizer. RNA was extracted from isolated glomeruli with TRIZOL™ according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Cell Culture and Transfection

A temperature-sensitive rat podocyte cell line, 2DNA1D7 was originally established by Dr Hidetake Kurihara (Juntendo University, Tokyo, Japan). The cell line was isolated by using nephrin expression as the marker from decapsulated glomeruli of tsA58 transgenic rats, and was cultured in DMEM/F-12 medium supplemented with 5% FCS and insulin-transferrin-selenium A (Invitrogen, Carlsbad, CA, USA). Cells were cultured at 33°C in a humidified CO_2 incubator (5% CO_2/95% air) and passaged for proliferation. The cells were transferred to 37°C for induction of differentiation. Owing to the fact that 2DNA1D7 cells proliferate slowly and have low transfection efficiency, normal rat kidney epithelial (NRK52E) cells were used in the experiments requiring higher transfection efficiency. NRK52E cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified CO_2 incubator (5% CO_2/95% air).

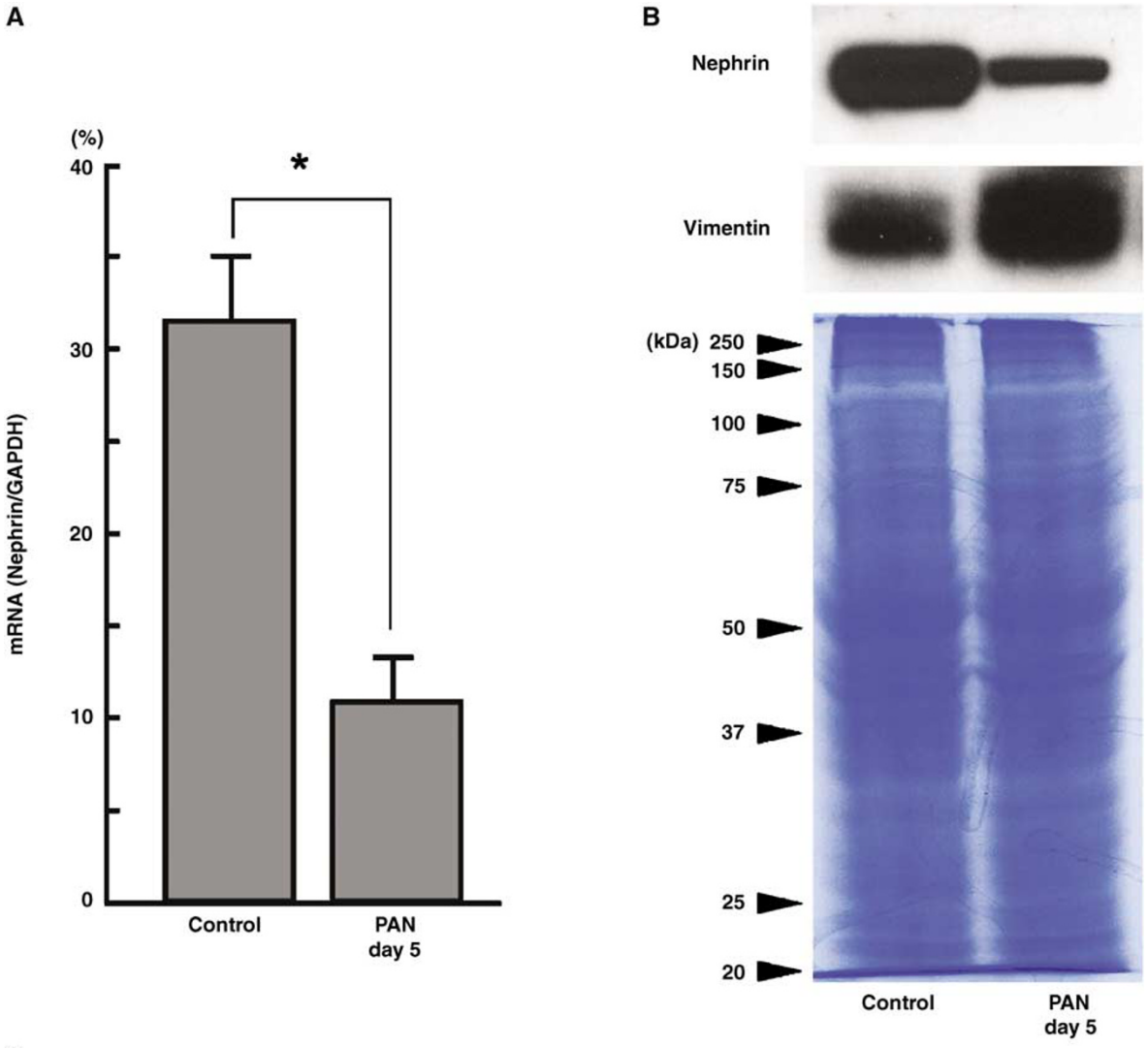
Immunoblot Analysis and Immunohistochemical Analysis

Antibodies against specific molecules were obtained as follows: HA epitope (Roche, Basel, Switzerland); Snail (AbCam, UK); nephrin (Santa Cruz Biotechnology, CA, USA); WT-1 (Santa Cruz Biotechnology, CA, USA); β-actin (Sigma, St Louis, MO, USA). An alternative nephrin-specific antibody was also developed in rabbits by immunization with the following peptides: CTLNVKYPAQKLWIEG as well as CEASSLPFELRGHLV and the characterization and purification procedures were performed as described previously.[18]

Figure 1 Nephrin is downregulated and vimentin upregulated in injured glomeruli. (**A**) RNA isolated from rat kidney glomeruli (control and PAN nephrosis day 5) were subjected to real time PCR analysis. Nephrin expression was normalized to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) expression. Nephrin was repressed at the mRNA level in PAN nephrosis glomeruli (*$P < 0.05$). (**B**) Nephrin was repressed at the protein level while vimentin expression was enhanced. Glomeruli were isolated at the same time point as (**A**) and examined by immunoblot analysis using anti-nephrin and anti-vimentin antibodies. For the loading control, the same sample was stained with coomassie brilliant blue because commonly used controls such as β-actin and α-tubulin were upregulated in the glomeruli of PAN nephrosis day 5 (data not shown). Two types of anti-nephrin antibodies were used for the experiments, and gave the identical result. Data using polyclonal antibodies that we prepared is shown. (**C**) Vimentin immunohistochemistry of control kidney (a) and PAN nephrosis day 5 kidney (b) are shown. Vimentin was predominantly expressed in podocytes and upregulated when injured.

In Situ Hybridization Analysis

Sections (4 μm thickness) were deparaffinized, rehydrated, and further fixed in 4% PFA/PBS for 20 min at room temperature (RT). The sections were sequentially incubated in 50 mM Tris · HCl (pH 7.4) containing 5 μg/ml of proteinase K and 5 mM of EDTA for 15 min at room temperature, in 0.2 N HCl for 10 min at RT, and in 0.25% acetic anhydride/0.1 M triethanolamine for 10 min at RT. The sections were



prehybridized with the hybridization buffer (4 × SSC/50% formamide/10% dextran sulfate/1 × Denharde solution/ 2 mM EDTA/denatured salmon sperm DNA (500 ng/ml)) for 1 h at 37°C. Hybridization was performed with 2.5 μg/ml of digoxigenin-labeled cRNA probes in the hybridization buffer at 50°C. Once hybridization was completed, the sections were washed in 2 × SSC at 50°C for 15 min and were incubated at 37°C for 10 min in 10 mM Tris · HCl (pH 7.5)/1 mM EDTA/ 0.5 M NaCl/ribonuclease A (20 μg/ml). Sections were washed twice in 2 × SSC at 50°C for 15 min and twice in 0.2 × SSC at 50°C for 15 min and digoxigenin was immunologically detected using DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Indianapolis, IN, USA). For fluorescent *in situ* hybridization, fluorescein-labeled nephrin cRNA and digoxigenin-labeled Snail cRNA were visualized with Tyramide Signal Amplification system according to the manufacture's instruction (Perkin Elmer, Fremont, CA, USA).

Quantitative Reverse Transcription-PCR

To quantify the expression of nephrin mRNA in kidney glomeruli and the podocyte cell line 2DNA1D7, real time SYBR-Green PCR analysis was performed. Briefly, 0.4 μg of RNA was converted to single-strand DNA with random primers (Invitrogen) and SuperScript II (Invitrogen). Each cDNA was mixed with 0.5 μM of forward and reverse primers, and 12.5 μl of GYBR Green Master Mix (Applied Biosystems, CA, USA). PCR was performed with Applied Biosystems 7700 real-time PCR system. The primers sets had the following sequences: Nephrin forward, 5′-GCTCCCAC CATCCGTGC-3′; Nephrin reverse, 5′-GACTATGTCCACA CAACCCCCA-3′; GAPDH forward, 5′-CTCTACCCACGG CAAGTTCAA-3′; GAPDH reverse, 5′-GGATGACCTTGCC CACAGC-3′. To quantify Wnt-2 expression in glomeruli, the same primer set described below was used.

Semiquantitative Analysis with RT-PCR

Differential expression of Wnt gene was confirmed by RT-PCR. Briefly, each cDNA was mixed with 2 μl of 10xPCR buffer (Takara, Tokyo, Japan), 1 μl of dNTP mixture (Takara), 1 μM of forward and reverse primers, and 0.1 μl of Takara rTaq. PCR was performed with GeneAmp PCR System 9700 (Applied Biosystems, CA, USA). The primer sets had the following sequences:

Wnt-1 forward, 5′-CTGCAGCTACTGAGCCGCAAG-3′;
Wnt-1 reverse, 5′-AGTTCCAGCGGCGGTTTCGGA-3′;
Wnt-2 forward, 5′-GGTCAGCTCTTCATGGTGGTACA TGAG-3′;
Wnt-2 reverse, 5′-CAATGGCACGCATCACGTCTGGGTGT-3′;
Wnt-2b forward, 5′-TGGAGGGCACTCTCAGACTTCC-3′;
Wnt-2b reverse, 5′-GCCTTGTCCAAGACACAGTAGT-3′;
Wnt-3 forward, 5′-CGCTGGAACTGTACCACCATAG-3′;
Wnt-3 reverse, 5′-TGACTGCGAAGGCTACACCAG-3′;
Wnt-4 forward, 5′-TCAGGTTGGCCACGCACTAAAGGA GAA-3′;
Wnt-4 reverse, 5′-AGTCTGGACTTGGCTCCAGGTACA CC-3′;
Wnt-5a forward, 5′-CTTCCGCAAGGTGGGCGATGC-3′;
Wnt-5a reverse, 5′-TTGCACAGGCGTCCCTGCGTG-3′.

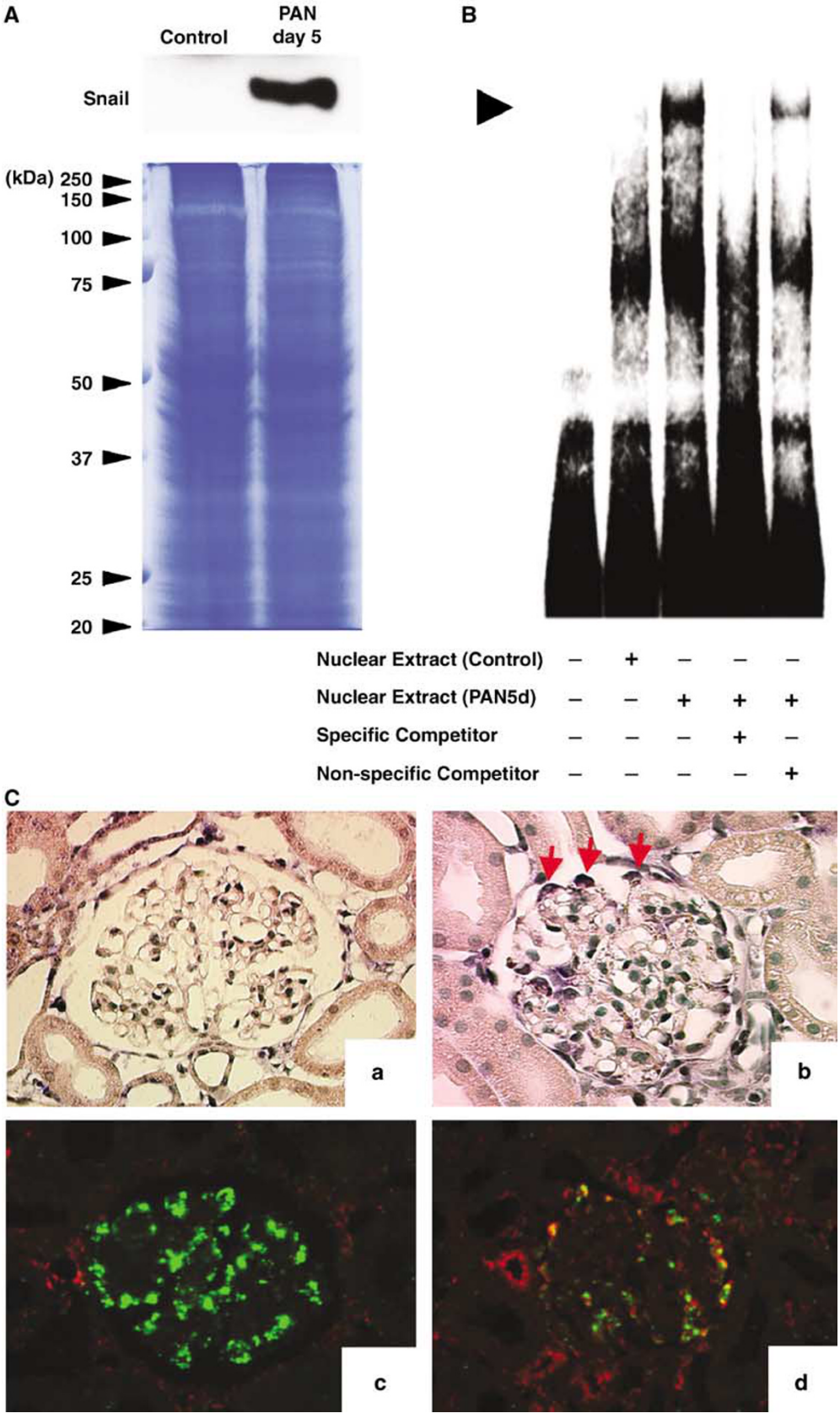
PCR products were separated on a 2.0% agarose gel, stained with ethidium bromide, and visualized using UV irradiation.

Assay for Transcriptional Regulation of Rat Nephrin Gene

Rat nephrin gene (−824 to + 1877 bp) was isolated from the rat tail genomic DNA by PCR with the following primers: 5′-AAAGGTACCAGATAAGCAGGCAGCAGGAGT-3′ and 5′-AAACTCGAGTTGCACACCTGGCTTCGGCCT-3′. The PCR product was cloned into *Kpn*I/*Xho*I-cut pGL3-SV40 vector (Promega, USA) and various fragments of rat nephrin gene (−824 to + 403 bp/ + 404 to + 1877 bp/ + 404 to + 1322 bp/ + 1323 to + 1877 bp) were subcloned into *Kpn*I/*Xho*I-cut pGL3-Basic vector by using PCR with following primers:

+ 403 bp reverse, 5′-AAACTCGAGTACTGGCAGCTTG GATTGTTGAG-3′; + 404 bp forward, 5′-AAAGGTACCAT GGGCGCTAAGAGAGTCACT-3′; + 1322 bp reverse, 5′-AAA CTCGAGCACTCTGGATGAAGGTGATG-3′; + 1323 bp forward, 5′-AAAGGTACCAGTGGGGGTGAACCGGTGGGAT-3′. For pGL3-SV40 constructs with + 404 to + 1877 bp/ + 404 to + 1322 bp/ + 1323 to + 1877 bp, which contain no core promoter region, segment −300 to −1 bp was inserted to the *Kpn*I site. Snail-dependent transcriptional regulation of nephrin gene was studied by a slightly modified luciferase assay.[18] pGL3-SV40 vector carrying various nephrin gene segments (2.5 μg) were introduced along with pRL-TK vector (50 ng) and pCAGGSneo HA-tagged Snail (2.5 μg) into 2×10^6 NRK52E cells and cultured in a six-well plate. After 24 h, both Firefly and Renilla luciferase activities were measured as described above. Firefly luciferase activities were normalized to the Renilla luciferase activities.

Figure 2 Snail is activated in injured podocytes. (**A**) Immunoblot analysis of Snail is shown. The same sample of Figure 1B was analyzed with anti-Snail antibody. There was a strong signal in the PAN nephrosis day 5 glomeruli, where the control showed no signal. For the loading control, the same sample was stained with coomassie brilliant blue. (**B**) EMSA for Snail is shown. Nuclear extracts from glomeruli of control rats and PAN nephrosis rats (day 5) were analyzed. The arrowhead indicates the binding activity, which was out-competed by a specific competitor. (**C**) *In situ* hybridization analysis was performed with normal rat kidney (a, c) and PAN nephrosis day 5 kidney (b, d) to elucidate the cell that expressed Snail. Snail was localized to injured podocytes but not to normal ones. Red arrows indicate the representative cells (b). For fluorescent *in situ* hybridization, fluorescein-labeled nephrin cRNA probe (green) and digoxigenin labeled Snail cRNA probe (red) was used. Podocyte marker nephrin was repressed in Snail expressing PAN nephrosis podocytes (d) compared to normal ones (c).


A
Control
PAN day 5
Snail
(kDa) 250
150
100
75
50
37
25
20
B
Nuclear Extract (Control) – + – – –
Nuclear Extract (PAN5d) – – + + +
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C
a
b
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ChIP Assay

The HA-tagged human Snail expression plasmid or an empty plasmid was transfected into 2DNA1D7 at 33°C with Amaxa Nucleofector™ (Amaxa Inc., Gaithersburg, MD, USA). Chromatin immunoprecipitation (ChIP) assay was performed as described.[29] Briefly, cells were incubated with 1% formaldehyde in PBS after 24 h culture at 37°C. The cells were washed twice with ice-cold PBS, and suspended in 0.1% SDS/0.1% sodium deoxycholate/1% Triton X-100/1 mM EDTA/0.5 mM EGTA/140 mM NaCl/10 mM Tris · HCl (pH8.0) containing protease inhibitors cocktail Complete™ (Roche Applied Science, Indianapolis, IN, USA). The resultant lysate was sonicated and immunoprecipitated with anti-HA antibody. Finally, DNA associated with the immunoprecipitated protein was evaluated by PCR with the following primers: forward, 5′-AGCTGGGCAGGAGTATG-3′; reverse, 5′-TCTGGATGAAGGTGATGT-3′.

Preparation of Nuclear Extract and EMSA

After the indicated treatment, kidneys were rinsed with ice-cold PBS and glomeruli were collected as described previously.[18] Nuclear protein from glomeruli was extracted with Nuclear Extraction Kit (Panomics, Redwood City, CA, USA) according to the manufacturer's instructions. Electrophoretic Mobility Shift Assay (EMSA) was performed with Panomics' EMSA Kit in combination with the probes, specific competitors and non-specific competitors for Snail (Panomics, Fremont, CA, USA) according to the manufacturer's instructions. Sequences of these nucleotides are not disclosed by the manufacturer.

Statistical Analysis

Statistical significance between experimental values was evaluated by a non-paired *t*-test. Significance was defined as $P<0.05$.

RESULTS

mRNA and Protein Product of Nephrin is Down-Regulated in Injured Podocytes

PAN-induced nephrosis (PAN nephrosis) in rats is a highly reproducible self-limiting disease with reversible podocytes injury. As our data using PAN nephrosis identifies day 5 of disease as the time point when the differential expression of mRNA reaches the maximum,[18] we employed normal rats and age-matched PAN nephrosis rats. In accordance with previous reports,[12,13,15,18] nephrin mRNA (Figure 1A) and protein product (Figure 1B) were markedly decreased in glomeruli of PAN nephrosis rats. In contrast, vimentin expression increased in the glomeruli of PAN nephrosis (Figure 1B) and was localized predominantly to podocytes (Figure 1C) and this was consistent with the report from Zou *et al*.[30] These results which are reminiscent of EMT because nephrin is a component of the cell–cell junction among adjacent podocytes prompted us to examine the involvement of the transcriptional regulator Snail in podocytes since Snail promotes EMT.[28]

Snail is Activated in Injured Podocytes and Represses the Transcription of Nephrin

Immunoblot analysis of glomeruli showed a striking upregulation of Snail in glomeruli of PAN nephrosis rats (day 5) (Figure 2A) whereas control rats did not express Snail (Figure 2A). We examined the DNA-binding activity of Snail by EMSA using nuclear extract of isolated glomeruli and confirmed the strong induction of Snail in glomeruli of PAN nephrosis (day 5) (Figure 2B). *In situ* hybridization analysis showed that Snail was localized to podocytes of PAN nephrosis (Figure 2Cb). In accordance with the result of immunoblot analysis (Figure 2A), mRNA of Snail was not observed in the podocytes of age control rats (Figure 2Ca). In addition to the induction of Snail on injured podocytes, fluorescent *in situ* hybridization displayed that nephrin was reduced in Snail expressing podocytes (Figure 2Cc, d).

By analogy with Snail-induced repression of E-cadherin,[28] we investigated whether Snail represses nephrin expression at the transcriptional level. Figure 3a represents the various segments of rat nephrin gene used for the following experiments with Firefly Dual luciferase assay system. In Figure 3a, $+1$ denotes the start of nephrin transcription. Each segment flanked by the Luciferase reporter gene was transfected with the pRL-TK control vector and with pCAGGSneo HA-tagged Snail or empty vector into a normal rat kidney epithelium cell line (NRK52E). The results are shown as the ratio of the transcriptional activity of Snail to the transcriptional activity of vector alone (Figure 3b). Snail repressed the transcriptional activity when the longest segment A was used. This repressor activity was found in the segment B2 that include the coding region of rat nephrin but not in the segment B1 that includes the promoter region of rat nephrin gene. Finally, we found that the segment C1 was responsible for the repressor activity of Snail on nephrin (Figure 3b). We attempted to further characterize the Snail binding segment in the segment C1 by using this assay, but were unsuccessful. However, we found five E-box motifs in the segment C1 to which Snail could potentially bind according to literature (Figure 3c).[27] All five E-box motifs may be necessary for the association between Snail and nephrin gene.

A rat podocyte cell line was used to prove that Snail binds to the genomic DNA of nephrin and represses nephrin transcription in podocytes. A temperature-sensitive rat podocyte cell line 2DNA1D7 was isolated from decapsulated glomeruli of tsA58 transgenic rats using nephrin expression as the marker. 2DNA1D7 was kept at 33°C for proliferation and then transferred to 37°C for differentiation. One of podocyte markers WT1 was constitutively expressed in the cells at both 33 and 37°C while nephrin was dramatically induced at 37°C (Figure 4a). We also confirmed that a podocyte cytoskeleton component α-actinin 4 was expressed at both 33 and 37°C by RT-PCR (data not shown). As

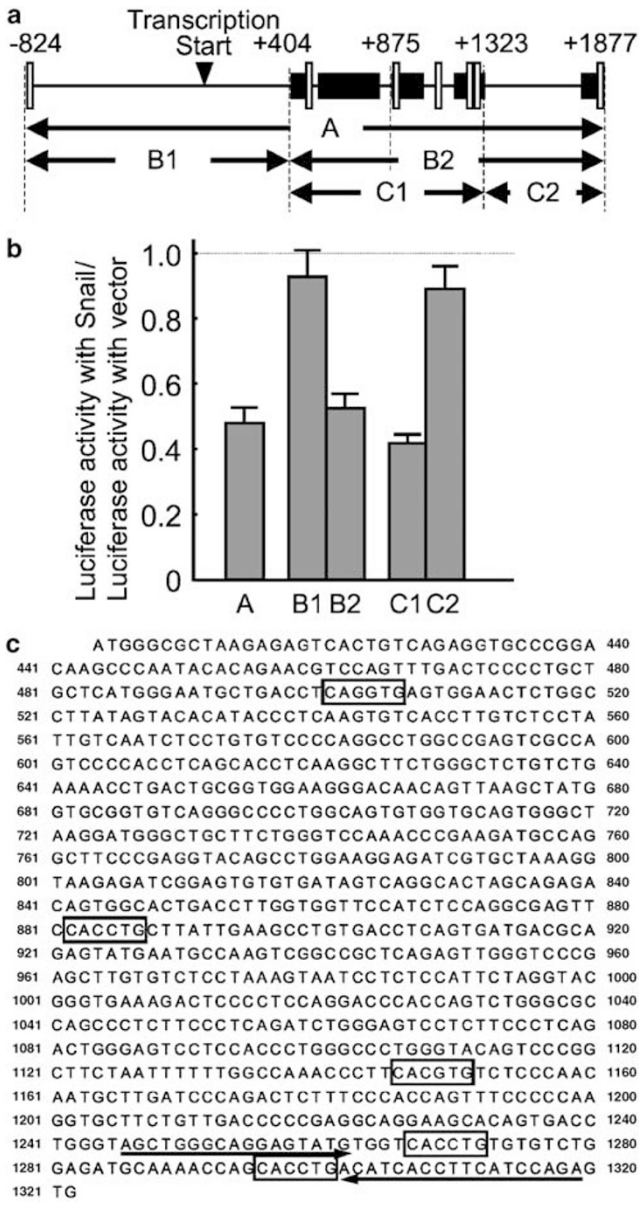


Figure 3 Snail represses nephrin transcription. (**a**) Segments of rat nephrin gene used for the following experiments are shown, where +1 denotes the start of nephrin transcription. Closed boxes indicate exons and open boxes indicate E-box motifs. (**b**) Repressor activity of each nephrin gene segment was assayed with Dual Luciferase Assay System. Each nephrin gene construct and pRL-TK control vector was co-transfected with Snail expression construct or empty control vector. The results are shown as the ratio of the transcriptional activity with Snail to the transcriptional activity with empty control vector. The segment C1 was responsible for nephrin repressor activity with Snail. (**c**) The sequence of segment C1 is shown. Boxed areas are E-box motifs, which are the putative binding sites of Snail. Arrows indicate the primer sets used for the following ChIP assay (Figure 4b).

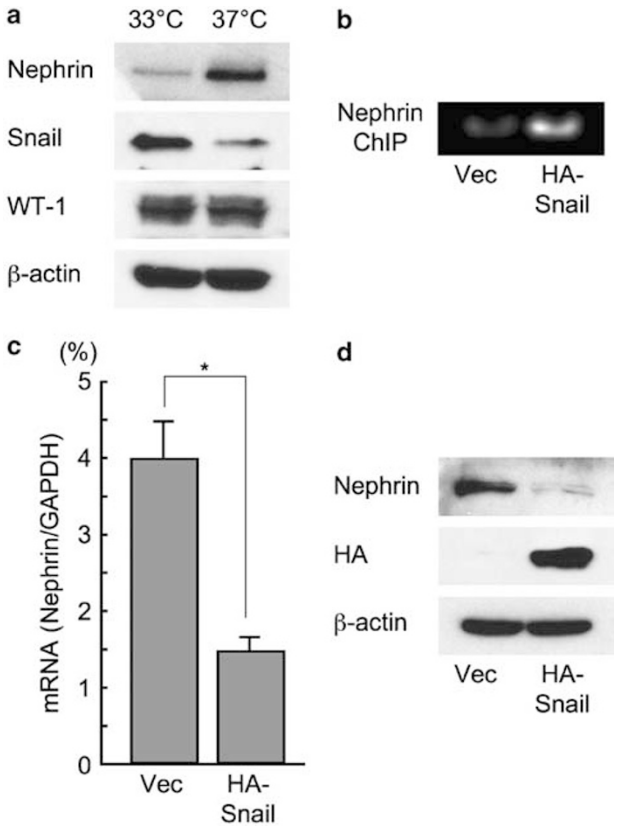
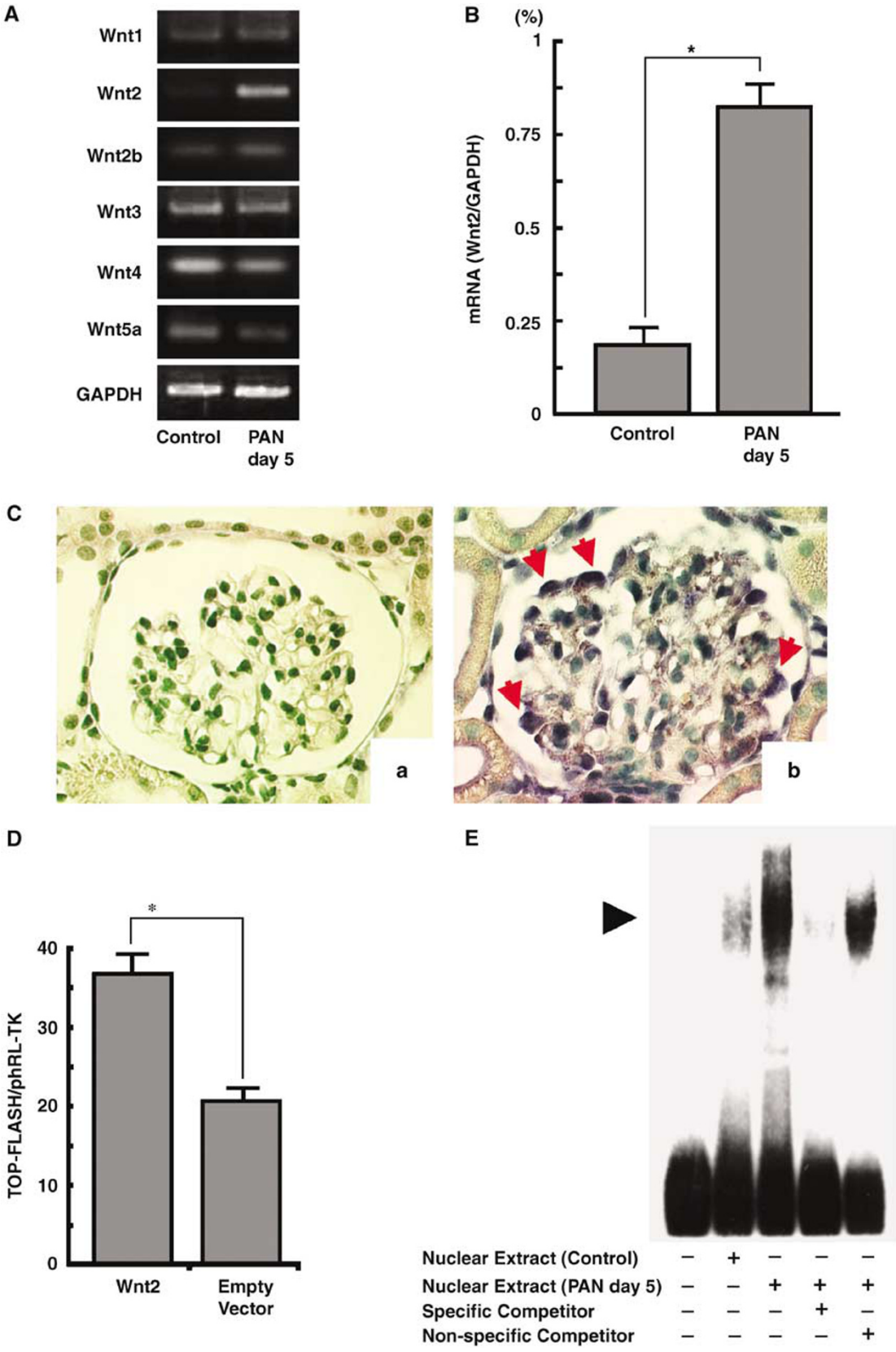


Figure 4 Snail represses endogenous nephrin in podocyte cell line 2DNA1D7. (**a**) 2DNA1D7 is a temperature-sensitive podocyte cell line, which was isolated by using nephrin expression as the marker from decapsulated glomeruli of tsA58 transgenic rats. This cell line was kept at 33°C for proliferation and at 37°C for differentiation. Podocyte specific markers were analyzed by immunoblot analysis. 2DNA1D7 cells at 37°C upregulated nephrin and downregulated Snail. (**b**) Snail bound to the segment C1 of the nephrin gene. HA-tagged Snail-expression construct or control vector was introduced into 2DNA1D7 cells. Then, chromatin immunoprecipitates prepared with anti-HA antibody were subjected to PCR using the primer sets in Figure 3c (arrows). (**c**) Nephrin expression of 2DNA1D7 cells transfected with empty vector or HA-tagged Snail expression construct was analyzed by real-time PCR. Snail repressed nephrin transcription (*$P<0.05$). (**d**) The same sample in (**c**) was analyzed by immunoblot using anti-nephrin antibody. Endogenous nephrin was repressed by Snail overexpression.

expected, Snail was strongly expressed at 33°C but almost disappeared at 37°C (Figure 4a). ChIP assay using the primer sets shown in Figure 3c (arrows) demonstrated that overexpressed Snail bound to the segment C1 in the cells (Figure 4b). The experiment using the antibody against non-tagged rat Snail was not successful, possibly because only trace amounts of Snail were expressed at 37°C. Once over-expressed, however, Snail repressed the transcription of nephrin in the cells (Figure 4c), and was accompanied by a significant decrease in the expression of the nephrin protein product (Figure 4d).

Snail Stabilizing Mechanism is also Activated in Injured Podocytes

Our results indicate that Snail plays a critical role in regulating the phenotype of podocytes. Therefore, we were interested in the mechanism by which Snail expression levels are regulated in podocytes. It is known that Snail is strictly


A
Wnt1
Wnt2
Wnt2b
Wnt3
Wnt4
Wnt5a
GAPDH
Control
PAN day 5
B
(%)
1
0.75
0.5
0.25
0
mRNA (Wnt2/GAPDH)
*
Control
PAN day 5
C
a
b
D
40
30
20
10
0
TOP-FLASH/phRL-TK
*
Wnt2
Empty Vector
E
Nuclear Extract (Control) – + – – –
Nuclear Extract (PAN day 5) – – + + +
Specific Competitor – – – + –
Non-specific Competitor – – – – +

controlled by different signaling molecules such as epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF)-β, bone morphogenic protein (BMP), Notch, parathyroid hormone-related peptide (PTH(rP)), and Wnt.[28] Recently, Zhou *et al*[31] and Yook *et al*[32] have demonstrated that the stability of Snail protein is regulated by glycogen synthase kinase 3 (GSK3), a downstream component of the Wnt canonical pathway. GSK3-dependent phosphorylation of Snail leads to its degradation via the ubiquitin pathway.[31,32] Based on this knowledge, we further investigated whether the signal transduction pathway from Wnt to GSK3 participates in the regulation of Snail in podocytes.

Semiquantitative RT-PCR analysis for the Wnt family revealed that Wnt-2, a member of the Wnt canonical pathway, was most remarkably upregulated in glomeruli of PAN nephrosis rats (day 5) as compared to glomeruli of control rats (Figure 5A, B). Wnt-4 and Wnt-5a—non-canonical Wnt proteins—were downregulated, and Wnt-1, Wnt-2b, and Wnt-3 did not change significantly (Figure 5A). During the time course of PAN nephrosis, mRNA for Wnt-2 reached the highest level on day 5 (not shown). *In situ* hybridization analysis using PAN nephrosis rats (day 5) showed that Wnt-2 transcript was localized to podocytes (Figure 5Cb).

As previously reported, Wnt-2 activates the Wnt canonical pathway where GSK3β is inactivated and β-catenin, a well-known substrate of GSK3β is unphosphorylated.[33–35] The unphosphorylated form of β-catenin accumulates in the nucleus and enhances the transcription of target genes in association with the T-cell factor/lymphoid enhancer factor (TCF/LEF) family.[36] Indeed, using TOP-FLASH assay, a Wnt canonical luciferase reporter, we could confirm the activation of Wnt canonical pathway by Wnt-2 (Figure 5D). Furthermore, EMSA using nuclear extract of isolated glomeruli displayed strong activation of the TCF/LEF complex in glomeruli of PAN nephrosis (day 5) (Figure 5E). This result is consistent with the report that Cyclin D1, a representative downstream molecule of the TCF/LEF complex transcription, was upregulated in podocytes of PAN nephrosis.[37] These results suggested that the upregulation of Wnt-2 in injured podocytes leads to the inhibition of GSK3β with the subsequent stabilization of Snail which represses nephrin transcription.

We confirmed by the use of BIO (100 nM)[38] or LiCl (50 mM) which are inhibitors for GSK3, that the activity of GSK3β affects the expression level of nephrin to activate the Wnt canonical pathway in NRK52E and 2DNA1D7 cells. Snail was expressed at a significant level under the described experimental condition (data not shown). Without the overexpression of Snail as shown in Figure 3b, both BIO and LiCl markedly suppressed the activity of nephrin transcription through the segment C1 (Figure 6a, b). BIO and LiCl also decreased the amount of nephrin protein product when applied to 2DNA1D7 cells (Figure 6c).

BIO treatment of PAN nephrosis rats exacerbated proteinuria (Figure 7a) along with a further decrease in nephrin and increase in Snail expression (Figure 7b). Transcriptional repression of nephrin gene was at least partly responsible for the BIO-mediated decrease in the nephrin protein product (Figure 7c). It should be noted, however, that BIO treatment alone did not induce the expression of Snail or affect the levels of nephrin protein product and it's mRNA (Figure 7b, c).

DISCUSSION

Our data show that the levels of Snail mRNA and protein are upregulated in podocytes in response to injury (Figure 2), and that Snail acts as a transcriptional repressor of nephrin

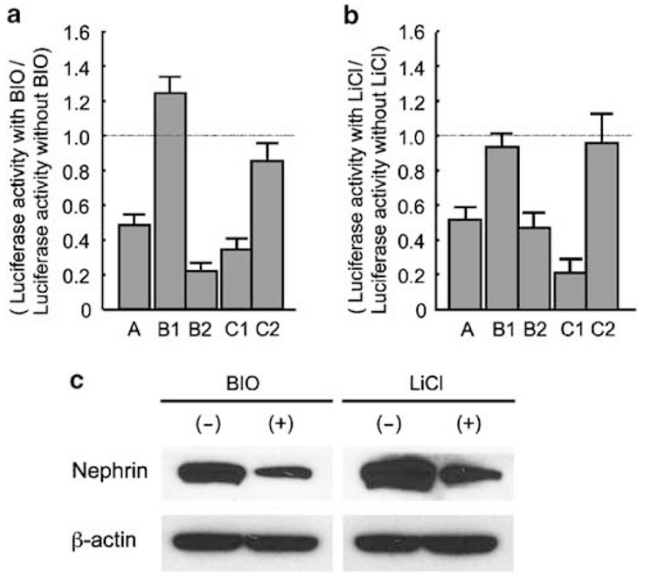


Figure 6 GSK3 inhibitors decrease the expression level of nephrin *in vitro*. Repressor activity of each nephrin gene segment, used in Figure 3, in the presence of GSK3 inhibitor BIO (**a**) or LiCl (**b**) is shown. Each nephrin gene segment was co-transfected with pRL-TK to NRK52E cells. The value of 'Firefly luciferase/Renilla luciferase' in the absence of the inhibitor was designated as 1.0. (**c**) Immunoblot analysis of nephrin in 2DNA1D7 cells treated with BIO or LiCl at 37°C. β-actin was used as the control.

Figure 5 Wnt pathway is activated in injured podocytes. (**A**) mRNA levels of various Wnt members are shown. RT-PCR products were applied to agarose gel and stained with ethidium bromide. RNA was prepared from glomeruli of control rats (*Cont*) or PAN nephrosis rats (day 5) (*PAN*). (**B**) Wnt-2 mRNA was quantitatively analyzed by real time PCR. (**C**) Injured podocytes expressed Wnt-2. Control (a) or PAN nephrosis (day 5) kidneys (b) were analyzed by *in situ* hybridization. The red arrows indicate representative cells. (**D**) The activation of Wnt canonical pathway by Wnt-2 was confirmed using TOP-FLASH luciferase assay ($^*P<0.05$). (**E**) EMSA for the TCF/LCF complex, a down stream of Wnt canonical pathway, is shown. Nuclear extract from glomeruli of control rats or PAN nephrosis rats (day 5) was used. The arrowhead indicates the binding activity.

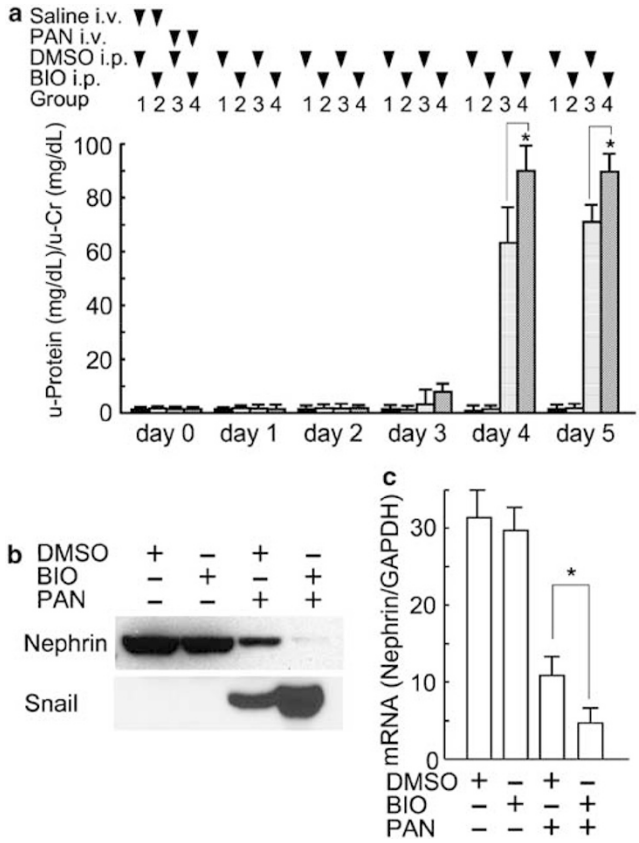


Figure 7 Inhibition of GSK3 *in vivo* exacerbates proteinuria and leads to the suppression of nephrin. (**a**) Effects of GSK3 inhibition on proteinuria are shown. A GSK3 inhibitor BIO or vehicle (DMSO) was intraperitoneally administrated once daily for 6 days (day 0–5) to rats with or without intravenous injection of PAN on day 0. Rats were divided into four groups ($N=4$ in each group). *Groups 1 and 3*, DMSO; *Groups 2 and 4*, BIO; *Groups 1 and 2*, saline; *Groups 3 and 4*, PAN. Group 4 excreted much higher amounts of urinary protein than Group 3 on days 4 and 5 ($^*P<0.05$). Proteinuria was measured as the ratio of urinary protein concentration (mg/dl) to urinary creatinine concentration (mg/dl). (**b**) Effects of GSK3 inhibition on the expression levels of nephrin and Snail are shown. Glomerular samples were collected from each group on day 5 and evaluated by immunoblot analysis. (**c**) Effects of GSK3 inhibition on nephrin mRNA are shown. Glomeruli were isolated at the same time point as (**b**) and subjected to real-time PCR ($^*P<0.05$).

(Figure 4) through the segment C1 (Figure 3). This pathway at least partly accounts for the phenotypic change of podocytes from normal status to pathological status. The zinc-finger domain of Snail may function as the nuclear localization signal as reported.[39] In genes regulated by Snail,[40] there is great variation regarding the location of the E-box motifs. In accordance with the segment C1, Snail-mediated repression of claudin-7 is derived mainly from E-box motifs which are located downstream from the putative transcription start point.[24] At present, all five E-box motifs in the segment C1 are candidates for the association with Snail, but the association of these E-box motifs with non-tagged native Snail remains to be confirmed.

Our data conclusively demonstrates that GSK3β is responsible for the accumulation of Snail in podocytes during PAN nephrosis (Figures 6 and 7). Changes in the activity of the Wnt canonical pathway at least partly account for the inactivation of GSK3β in podocytes (Figure 5). Nevertheless, how the transcription of Snail is initiated in response to injury is unknown. The fact that BIO treatment of normal rats neither induced Snail nor exacerbated proteinuria while BIO treatment of PAN nephrosis rats further upregulated Snail protein product and exacerbated proteinuria (Figure 7) indicates that GSK3β-mediated protein stabilization of Snail does not matter under normal conditions. In breast and skin epithelial cells, inhibition of GSK3 induces transcription of Snail.[41] Therefore, other key events may need to precede the transcription of Snail in injured podocytes. NF-κB promotes Snail transcription[41] and DNA-binding activity of NF-κB is stimulated in cultured podocytes under conditions of increased protein load.[42] It is plausible that both the activation of NF-κB and the inactivation of GSK3β are essential for the accumulation of Snail in podocytes. Snail protein product is also stabilized by p21-activated kinase 1 (PAK1) and by a zinc-finger transporter, LIV1.[31,32,43,44] Currently, we have no data to indicate whether PAK1 and/or LIV1 are involved in the regulation of Snail in podocytes.

The Wnt canonical pathway is not active in normal podocytes (Figure 5). This implies that GSK3 is constitutively active under normal condition. As BIO treatment alone does not induce proteinuria (Figure 7), the biological significance of constitutive activation of GSK3 in normal podocytes remains unknown. By contrast, it is still possible that GSK3 may help podocytes maintain the integrity of slit diaphragm by degrading trace amount of Snail protein. We believe this issue is worthy of further investigation.

In this study, we revealed the role and regulatory mechanism of Snail in injured podocytes and that Wnt-2 and GSK3 are involved in the activation of Snail under pathological conditions.

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