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Snail1 is involved in the renal epithelial–mesenchymal transition

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Abstract

The pathological significance of the tubular epithelial–mesenchymal transition (EMT) in kidney diseases is becoming increasingly recognized, and the transcription factor Snail1 plays a critical role in EMT. The results of this study show that Snail1 mRNA and protein were upregulated in the tubular epithelial cells of the obstructed kidneys in a rat model of unilateral ureteral obstruction and in human proximal tubule HKC-8 cells treated with TGF-β1. Glycogen synthase kinase-3β (GSK-3β) regulates the Snail1 level by degrading Snail1 protein. The level of the phosphorylated inactive form of GSK-3β was increased in the tubular epithelial cells of the obstructed kidney. TGF-β1 increased the phosphorylated form of GSK-3β in HKC-8 cells, and inhibition of GSK-3β by the selective inhibitors lithium and TDZD-8 caused Snail1 protein to accumulate. This study demonstrated that Snail1 is involved in renal tubular EMT and that TGF-β1 regulates Snail1 at the transcription and protein degradation levels.

Keywords: Obstructive nephropathy; Renal fibrosis; Renal tubular epithelial cells; Epithelial–mesenchymal transition; Glycogen synthase kinase-3β; TGF-β1; Snail1

The pathological significance of the tubular epithelial–mesenchymal transition (EMT) in renal fibrosis is becoming increasingly recognized [1]. Renal tubular EMT is a process in which renal tubular cells lose their epithelial phenotype and acquire characteristic features of mesenchyme. The tubular cells undergoing EMT may disrupt the basement membrane and invade the interstitium [2]. It has been hypothesized that the source of the myofibroblasts that reside in the interstitium and produce the extracellular matrix are the renal tubular cells that undergo EMT. Iwano et al. [3] reported that a large proportion (36%) of the interstitial fibroblasts in obstructed kidneys are derived from proximal tubular cells, indicating a far greater contribution of the EMT pathway to renal fibrosis than previously thought. However, since the molecular mechanism of renal tubular EMT has remained elusive, it is essential to identify the molecular events involved in the induction of EMT in this disease process in order to understand the mechanisms underlying the pathogenesis of renal fibrosis.

Genes of the *Snail* family encode the zinc finger proteins that have been reported to play pivotal roles in EMT during embryonic development and cancer metastasis [4]. Snails bind to specific DNA sequences called E-boxes in the promoter of the E-cadherin gene and of other genes and repress their transcription. Since E-cadherin is a major cell–cell adhesion molecule in epithelial cells, down-regulation of E-cadherin expression is assumed to be responsible for EMT. The mammalian *Snail* gene family includes *Snail1* (previously *Snail*), *Snail2* (previously *Slug*), and *Snail3*. One of them, *Snail1*, has been investigated extensively.

Snail1 activity is regulated by various signaling pathways at multiple levels [4]. Glycogen synthase kinase-3β (GSK-3β) has recently been demonstrated to regulate the Snail1 level by phosphorylation, β-TrCP-directed ubiquitination, and proteasomal degradation [5]. GSK-3β is a serine/threonine kinase that regulates a diverse array of cell

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functions. GSK-3β binds to and phosphorylates Snail1 protein at two consensus motifs to dually regulate its function [5]. Phosphorylation of the first motif regulates its β-TrCP-mediated ubiquitination, and phosphorylation of the second motif controls its subcellular localization. GSK-3β is normally active in cells, and it is inactivated by phosphorylation at its Ser9 residue. GSK-3β inactivation by phosphorylation stabilizes Snail1 protein and increases its levels in the nucleus.

Since Snail1 has been demonstrated to be involved in EMT in development and cancer metastasis, Snail1 is presumed to be involved in the tubular EMT process in chronic kidney diseases. In this study, we show upregulation of Snail1 in the tubular cells of the kidney on the obstructed side in a model of unilateral ureteral obstruction (UUO) and in cultured renal tubular cells treated with TGF-β1. GSK-3β is phosphorylated in the tubular cells of UUO kidneys and in cultured renal tubular cells treated with TGF-β1, and inhibition of GSK-3β leads to a decrease in the degradation of Snail1 protein. The results of this study indicate that TGF-β1 regulates Snail1 at the level of gene expression and protein degradation in renal tubular EMT.

Materials and methods

Animal model. UUO was performed in male Sprague–Dawley rats (Charles River Laboratories Japan, Inc., Yokohama, Japan) as described previously [6]. In brief, under general anesthesia the left ureter was ligated at two sites and transected between them. Rats were sacrificed under anesthesia 1, 3, 7, 14, and 21 days after surgery, and their right kidneys were used as intraindividual controls. Cross sections of each kidney were fixed in 10% neutral-buffered formalin for paraffin sections, and the remaining portion of each kidney was snap-frozen in liquid nitrogen for RNA and protein extraction. All procedures in the animal experiments complied with the standards in the Guidelines for the Care and Use of Laboratory Animals of Keio University School of Medicine.

Semi-quantitative RT-PCR and real-time PCR. Total RNA was extracted from whole kidney tissue with TRIzol reagent (Invitrogen Corp., Carlsbad, CA) or from cultured cells with an RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan). For semi-quantitative RT-PCR analysis, the number of PCR amplification cycles was determined in the exponential phase. The 18S ribosomal RNA (rRNA) level was used to standardize the mRNA level of the target genes. Real-time PCR was performed with a TaqMan ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) as described previously [7]. The following oligonucleotide primers and probes were used: rat Snail1, 5′-ACCCACACT GGTGAGAAGCC-3′ (sense), 5′-TTCACATCCGAGTGGGTCTG-3′ (antisense), and internal fluorescence-labeled TaqMan MGB probe (FAM), 5′-CCCACTGCAACCGTG-3′; human Snail1, 5′-ACCCACAC TGGCGAGAAGCC-3′ (sense), 5′-TTGACATCTGAGTGGGTCTG-3′ (antisense), internal fluorescence-labeled TaqMan MGB probe (FAM) 5′-CCCACTGCAGCCGTG-3′. All data were normalized by using 18S rRNA as an endogenous control. Each sample was tested in duplicate.

Immunoblotting. Whole kidney tissues or cultured cells were homogenized in RIPA buffer supplemented with proteinase inhibitors, and the supernatant was collected after centrifugation. Samples were subjected to SDS–PAGE, and transferred to a membrane. The membranes were probed with primary antibodies in PBS with 0.1% Tween 20 and then with peroxidase-conjugated secondary antibodies. The monoclonal Snail1 antibody was kindly provided by Dr. H. Höfler (Institut für Pathologie, Technische Universität München, Germany) [8]. The antibody against actin was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-GSK-3β (Ser9) antibody (Cell Signaling Technology, Inc., Danvers, MA) detects endogenous levels of GSK-3β only when phosphorylated at serine 9, and anti-GSK-3β antibody (Cell Signaling Technology, Inc.) detects endogenous levels of total GSK-3β protein.

Cell culture. HKC-8 human kidney tubular epithelial cells were kindly provided by Dr. L. Racusen of Johns Hopkins University [9] and cultured in DMEM/F12 with 10% FBS. The cells were switched to medium supplemented with 0.5% FBS for 24 h and treated with TGF-β1 (R&D Systems, Inc., Minneapolis, MN, USA), actinomycin D, cycloheximide, MG-132, LiCl (Sigma–Aldrich, St. Louis, MO), or TDZD-8 (Merck Biosciences Ltd., Beeston, Nottingham, UK) for various time periods and then collected for further analysis.

Immunohistochemical analysis. Paraffin sections were deparaffinized and hydrated, and the antigen was retrieved by boiling for 10 min in 10 mM citric acid, pH 6.0. The sections were incubated overnight at 4 °C with the primary antibody, either polyclonal anti-mouse Snail1 antibody (kindly provided by Dr. E. Fuchs, The Rockefeller University, New York) [10] or anti-phospho-GSK-3β (Ser9) antibody, then with the biotinylated secondary antibody for 1 h at room temperature, and finally with avidin–biotin horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame Inc., CA). The chromogen substrate was diaminobenzidine, and the sections were counterstained with hematoxylin.

Statistical analysis. Statistical significance was evaluated by Student's *t*-test or by one-way analysis of variance followed by a Tukey–Kramer multiple comparison test, as appropriate. Statistical significance was defined as $P < 0.05$. All values are expressed as means ± SEM.

Results

Snail1 expression during maturation of the kidney

First, we investigated expression of Snail1 in the fetal kidney and the adult kidney. RT-PCR revealed abundant Snai1 mRNA expression in the developing kidney, but a very low level of Snail1 mRNA in the normal adult kidney (Fig. 1A). The pattern of Snail1 expression coincided with that of a mesenchymal marker, vimentin. An immunohistochemical study at embryonic day 17 revealed that the Snail1 protein was predominantly expressed in the nuclei of metanephric mesenchymal cells surrounding the ureteric buds in the developing kidney, and there was no staining in the ureteric buds (Fig. 1B). There was no significant staining in normal adult kidney.

Induction of Snail1 in the tubular epithelial cells of obstructed kidneys

We used the most common model of tubulointerstitial disease, the UUO model, to investigate the function of Snail1 under pathological conditions. The kidneys of rats subjected to ureteral obstruction developed conspicuous evidence of tubulointerstitial damage. The non-obstructed contralateral kidneys did not show any morphological alterations. Changes in expression of Snail1 mRNA after UUO were quantified by a real-time PCR method, and Snail1 mRNA expression was significantly increased in the obstructed kidneys in comparison with the contralateral kidneys (Fig. 1C). Comparison with the sham-operated kidneys showed no increase in Snail1 mRNA in the UUO contralateral kidneys.

Snail1 protein was localized by an immunohistochemical technique. No Snail1 protein was detected in the

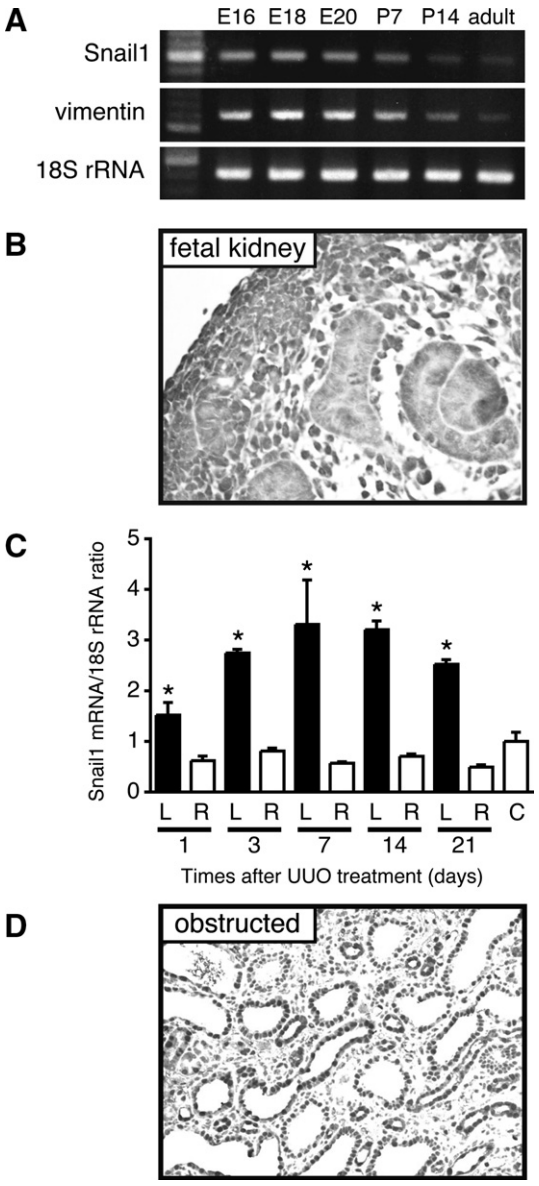

Fig. 1. Snail1 expression in the developing kidney and kidneys affected by ureteral obstruction. (A) Semi-quantitative-PCR analysis of *Snail1* and vimentin transcripts during kidney development. Reverse transcription-PCR (RT-PCR) was performed on RNA extracted from the kidneys of rats at embryonic day (E) 16, E18, E20, postnatal day (P) 7, P14, and from an adult kidney. RT-PCR products of 18S rRNA in the same sample are shown as a control. (B) Immunohistochemical localization of Snail1 protein in developing rat kidney at E17. (C) Expression of *Snail1* mRNA in the kidneys affected by ureteral obstruction. Total RNA was extracted from the unobstructed right kidney (R) and obstructed left kidney (L) at the times indicated after the UUO operation, and from the kidneys of a sham-operated rats (C). mRNA levels quantified by real-time PCR were normalized to an endogenous control 18S ribosomal RNA (rRNA) and are shown as ratios to the value in the control kidneys of sham-operated rats. Values are means ± SEM for four animals. $^{*}P < 0.05$ vs C. (D) Immunohistochemical localization for Snail1 protein in obstructed kidneys at 7 days after the UUO operation.

contralateral unobstructed kidneys (data not shown), whereas intense nuclear staining for Snail1 protein was observed in the tubular epithelial cells and weaker staining was observed in the nuclei of the cells that appeared to be fibroblasts in the fibrotic interstitium of the UUO kidneys (Fig. 1D). The most intense signals were observed in the tubular epithelial cells of dilated tubules.

TGF-β1 increased Snail1 expression in cultured renal epithelial cells

Because Snail1 expression was shown to be predominantly induced in the tubular cells of injured kidneys, we investigated the function of Snail1 in the cultured human renal proximal cell line HKC-8 [9]. Since TGF-β1 has been identified as an important molecular player in EMT both in vitro and in vivo [11], Snail1 expression was investigated in HKC-8 cells treated with TGF-β1. Expression of Snail1 mRNA in renal tubular cells was induced as early as 1 h after the addition of 3 ng/ml of TGF-β1, and the induction was sustained until 48 h (Fig. 2A). The induction of Snail1 mRNA was dose-dependent (Fig. 2B). The only profibrotic cytokine that significantly increased the expression of Snail1 mRNA was TGF-β1. EGF, PDGF, and TNF-α had no effect on gene expression of Snail1 in HKC-8 cells (data not shown). RNA and protein synthesis inhibitors were used to further examine the mechanism of the induction (Fig. 2C). Inhibition of RNA synthesis with actinomycin D 5 μg/ml abolished the induction of Snail1 mRNAs by TGF-β1 (3 ng/ml) in HKC-8 cells, but inhibition of protein synthesis with cycloheximide 10 μg/ml had no effect on induction of Snail1 mRNA by TGF-β1. This showed that the induction of Snail1 mRNA by TGF-β1 is consistent with a direct transcriptional effect. Snail1 was upregulated by TGF-β1 at the protein level, and the upregulation was paralleled by down-regulation of E-cadherin protein (Fig. 2D). Forced expression of Snail1 has been reported to induce EMT in various epithelial cell lines, including renal distal tubular cells (MDCK) [12]. We also observed down-regulation of E-cadherin expression in HKC-8 cells transfected with a plasmid coding Snail1 (data not shown).

Regulation of Snail1 by inactivation of GSK-3β in renal epithelial cells

Snail1 levels have recently been shown to be regulated by protein degradation, and GSK-3β has been found to play an important role in Snail1 degradation [5]. GSK-3β is a constitutively active kinase whose activity is inhibited by phosphorylation of the Ser9 residue, and phosphorylation of the Ser9 residue of GSK-3β is therefore thought to increase Snail1 protein stability.

To determine whether inactivation of GSK-3β in tubular cells is involved in the etiology of renal fibrosis, we investigated phosphorylation of the Ser9 residue of GSK-3β in the obstructed kidneys with an antibody that specifically recognizes GSK-3β phosphorylated at Ser9, and the immunoblot analysis showed an increased phospho-GSK-3β Ser9 level in the obstructed kidneys (Fig. 3A). Although, the total GSK-3β level was increased in the UUO kidneys, the increase in the phosphorylated form was greater. The

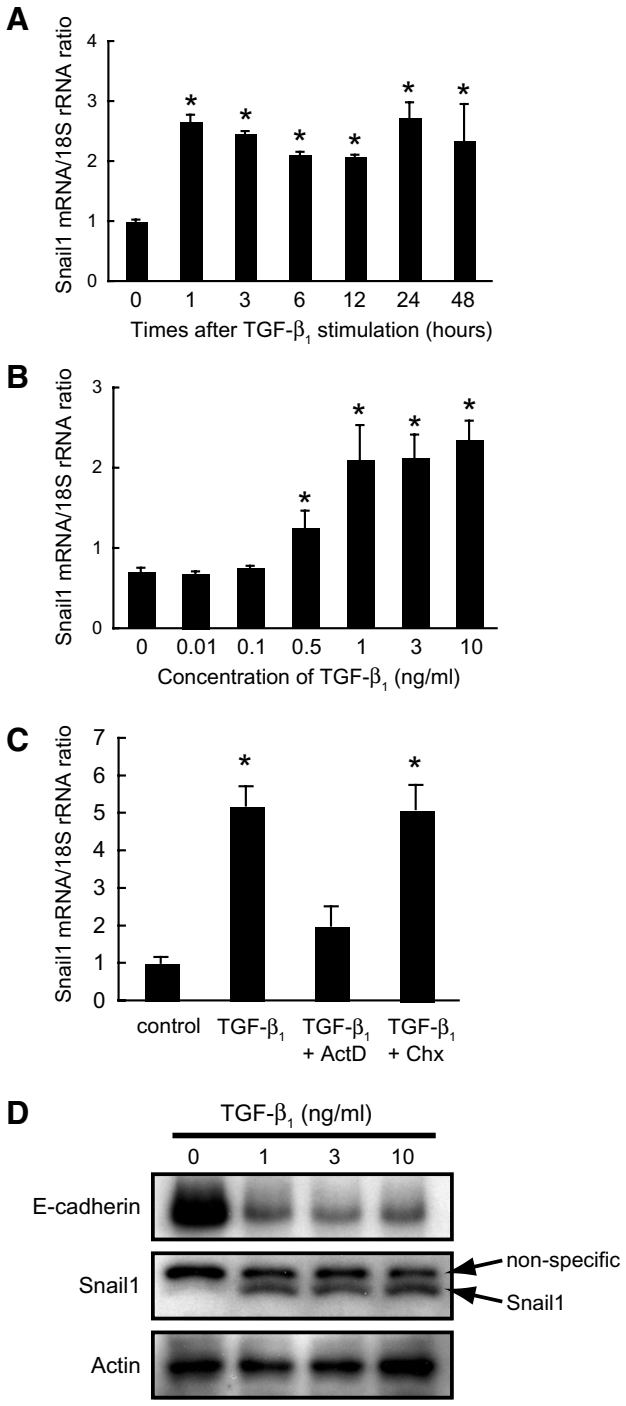

Fig. 2. TGF-β1 increased Snail1 expression in cultured renal epithelial cells. (A) Total RNA was extracted from human kidney tubular epithelial (HKC-8) cells at the times indicated after TGF-β1 (3 ng/ml) treatment. (B) HKC-8 cells were incubated with increasing amounts of TGF-β1 for 24 h. (C) Effect of actinomycin D (ActD) and cycloheximide (Chx) on TGF-β1-induced Snail1 mRNA in HKC-8 cells. HKC-8 cells were treated with TGF-β1 (3 ng/ml) for 24 h followed by ActD (5 μg/ml) or Chx (10 μg/ml) for 1 h. mRNA levels quantified by real-time PCR were normalized to an endogenous control 18S ribosomal RNA (rRNA) and are shown as ratios to the value at 0 h (A) or in the absence of TGF-β1 (B and C). The data shown are means ± SEM for four independent experiments. $^{*}P < 0.05$ vs control. (D) Immunoblots of E-cadherin, Snail1, and actin with proteins extracted from HKC-8 cells treated with increasing amounts of TGF-β1 for 24 h.

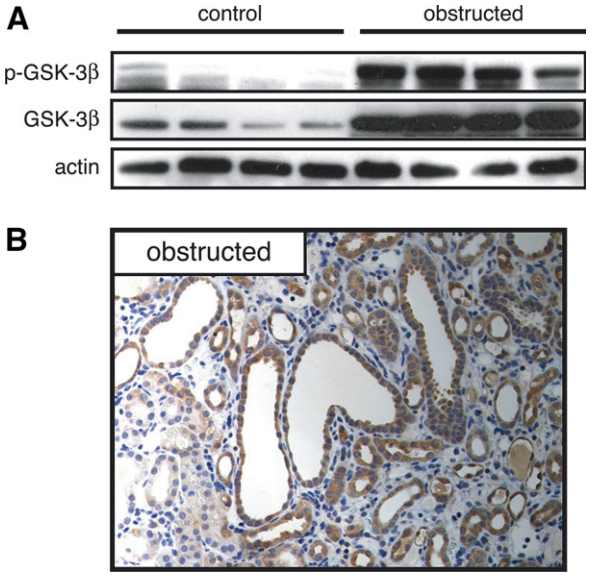

Fig. 3. Inactivation of GSK-3β in the tubular epithelial cells of obstructed kidneys. (A) Immunoblot of proteins extracted from obstructed and contralateral unobstructed (control) kidneys of four UUO rats at day 7. An antibody that specifically recognizes phosphorylated GSK-3β at the Ser9 residue, an anti-GSK-3β antibody, or an anti-actin antibody was used. (B) Immunohistochemical localization of phosphorylated GSK-3β at Ser9 in an obstructed kidney 7 day after the UUO operation.

phosphorylated GSK-3β protein was largely limited to the tubular epithelial cells of the obstructed kidneys (Fig. 3B). No immunoreactivity was detected in the contralateral unobstructed kidneys (data not shown).

Next, we investigated whether GSK-3β is involved in regulating the Snail1 protein level in HKC-8 cells. Treatment with TGF-β1 increased the level of phosphorylated form of GSK-3β (Fig. 4A). Since lithium is known to inhibit GSK-3β [13], we examined Snail1 expression after GSK-3β activity was inhibited with lithium. Lithium inhibits GSK-3β, in part, by increasing the phosphorylated form of GSK-3β. Lithium increased the level of the phosphorylated form of GSK-3β in HKC-8 cells, but had little effect on the total GSK-3β level (Fig. 4B). In accordance with increase in the phosphorylated GSK-3β, the level of Snail1 protein was markedly elevated. We also investigated the effect of TDZD-8, a synthetic chemical inhibitor of GSK-3β [14], and Snail1 protein accumulated when HKC-8 cells were incubated with TDZD-8 (Fig. 4C). These results clearly showed that inhibition of GSK-3β caused accumulation of Snail1, and that GSK-3β regulates Snail1 at the protein degradation level.

Discussion

The significance of renal tubular EMT has been recognized in chronic kidney diseases [1]. Snail1 is known to be a key regulator of EMT during development and cancer metastasis [4], inspiring us to investigate whether Snail1 is involved in renal tubular EMT. The results of this study provide evidence supporting a critical role of Snail1 in

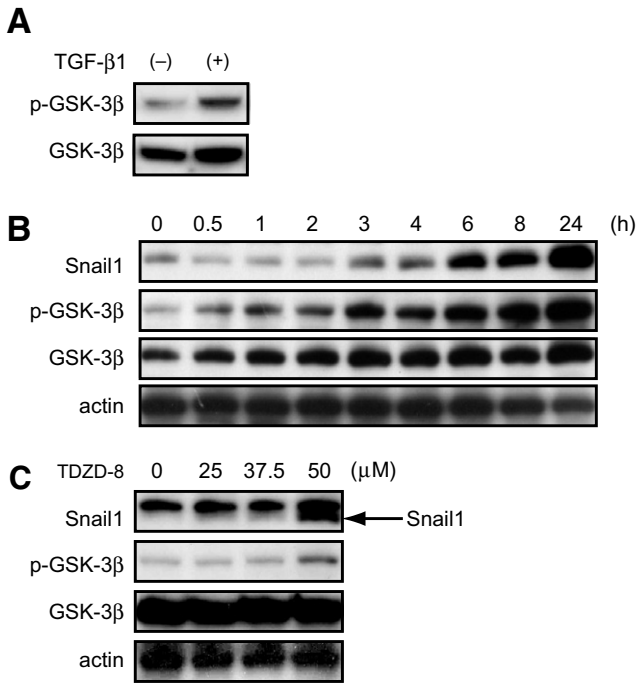

Fig. 4. Suppression of the GSK-3β stabilizes Snail1 protein in renal epithelial cells. (A) HKC-8 cells were exposed to 0 or 3 ng/ml TGF-β1 for 48 h. (B) HKC-8 cells were exposed to 20 nM LiCl, a specific GSK-3β inhibitor, for 0–24 h in the presence of 10 μM MG132. (C) HKC-8 cells were exposed to 0–50 μM TDZD-8, a specific GSK-3β inhibitor, for 24 h. Immunoblots were performed using antibodies against phospho-GSK-3β, total-GSK-3β, Snail1, and actin.

the renal tubular EMT process. Snail1 mRNA expression was enhanced in rat kidneys after UUO, and more intense signals were observed in renal tubular cells than in fibroblasts in the interstitium. Sato et al. showed specific localization of Snail1 mRNA in renal tubular epithelial cells 7 days after UUO [15], and the results of their in-situ hybridization study support our findings. We clearly showed the localization of Snail1 protein in diseased kidneys.

TGF-β1 has been identified as an important molecular player in EMT both in vitro and in vivo. Several studies have demonstrated a pivotal role of TGF-β1 signaling in tubular EMT in vivo. Sato et al. clearly showed that disruption of TGF-β1 signaling by gene targeting of Smad3, a key signaling intermediate downstream of the TGF-β1 receptors, blocked tubular EMT in a UUO model and ameliorated fibrosis following UUO [15]. Zeisberg et al. reported that systemic administration of recombinant human BMP-7 reverses TGF-β1-induced de novo EMT in a mouse model of chronic renal injury [16], and erythropoietin has been reported to decrease renal fibrosis in UUO by inhibition of TGF-β1-induced EMT [17]. We observed upregulation of Snail1 in HKC-8 cells treated with TGF-β1. Li et al. investigated Snail1 expression in HKC cells treated with a 2 ng/ml concentration of TGF-β1, but no protein was detected by immunoblotting, and they concluded that TGF-β1-triggered E-cadherin repression is independent of Snail1 [18]. Snail1 is highly unstable, susceptible to degradation by proteases, and has a short half-life of about 25 min [5]. Our success in detecting Snail1 is attributable to a rapid protein preparation procedure and the quality of the antibody provided by Dr. H. Höfler [8]. We validated the specificity of the antibody by using lysate of HEK293 cells transfected with Snail1.

Boutet et al. [19] recently reported that overexpression of Snail1 in the nucleus of epithelial cells causes EMT in vivo. They generated a transgenic mouse expressing a tamoxifen-inducible Snail1-ER construct in which the exogenous Snail1 is only active after nuclear transduction upon tamoxifen administration. Upon Snail1 activation, the tubular cells seemed to acquire a fibroblast-like morphology, and disrupted basement membranes and collagen I deposition were observed. Our results together with those of others demonstrated that Snail1 causes renal tubular EMT in vivo and in vitro and that pathological activation of Snail1 may contribute to renal fibrosis.

Another finding in our study was that Snail1 is regulated at the level of transcription and protein degradation by TGF-β1 in renal tubular EMT. Transcriptional upregulation of Snail1 was observed in UUO kidneys and HKC-8 cells treated with TGF-β1. TGF-β1, TGF-β2, EGF, FGF, HGF, BMPs, WNTs, and Notch have been reported to regulate Snail1 transcription [20], but the signaling systems vary with the cell type. TGF-β1-induced gene expression of Snail1 in HKC-8 cells, but EGF, PDGF, and TNF-α did not. Involvement of the MAPK and phosphatidylinositol 3-kinase signaling pathways has been demonstrated in TGF-β1-mediated induction of the *Snail1* promoter, and Sato et al. have clearly demonstrated that Smad3 is indispensable to TGF-β1-induced Snail1 gene expression in a study using Smad3-null mice [15].

Another mechanism of regulation of the Snail1 level has been shown to be governed at the protein degradation level. We observed elevated levels of GSK-3β phosphorylation in injured renal tubular cells in UUO kidneys that nearly coincided with the levels of Snail1 protein expression and also in tubular cultured HKC-8 cells. The elevated level of GSK-3β phosphorylation seemed to be correlated with Snail1 accumulation, since phosphorylated GSK-3β is the inactive form and cannot recruit a protein degradation system for Snail1.

To obtain more direct evidence, we measured the Snail1 protein level in HKC-8 cells treated with a GSK-3β specific inhibitor, lithium or TDZD-8. Lithium induced accumulation of Snail1 protein in a dose-dependent manner, and the increase in Snail1 level paralleled the level of the phosphorylated form of GSK-3β. TDZD-8 also increased the protein expression of Snail1. Based on all of these findings taken together, we concluded that inactivation of GSK-3β inhibits degradation of Snail1 protein in renal epithelial cells.

The results of the present study demonstrate that Snail1 mediates renal tubular EMT in renal fibrosis and that the Snail1 level is regulated at the transcription and protein degradation levels. The GSK-3β−Snail1 axis may be a suitable target for the treatment of chronic kidney diseases.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.07.146.

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