SnoN suppresses TGF-β-induced epithelial-mesenchymal transition and invasion of bladder cancer in a TIF1γ-dependent manner

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Abstract. The transcriptional regulator SnoN (also known as SKI-like proto-oncogene, SKIL), a member of the Ski family, has been reported to influence epithelial-mesenchymal transition (EMT) in response to TGF-β. In the present study, we investigated the role of SnoN in bladder cancer (BC). Differential expression of SnoN was not detected in BC tissues compared with that noted in adjacent non-cancerous tissues. SnoN was upregulated in response to TGF-β treatment, but had no effect on the TGF-β pathway, which may be explained by the low level of SnoN SUMOylation. TIF1γ, which catalyzes the SUMOylation of SnoN, was downregulated in BC tissues. Overexpression of TIF1γ restored the ability of SnoN to suppress the TGF-β pathway. Furthermore, TGF-β-induced EMT and invasion of BC cells were suppressed by TIF1γ in the presence of SnoN. Collectirely, our data suggest that SnoN suppresses TGF-β-induced EMT and invasion of BC cells in a TIF1γ-dependent manner and may serve as a novel therapeutic option for the treatment of BC.

Introduction

Bladder cancer (BC) is one of the most common cancers of the urinary system worldwide (1). Approximately 90% of BCs are urothelial cell carcinomas with an epithelial origin (2). Muscle-invasive BC occurs in ~1/3 of patients and is associated with a poor prognosis, with a 5-year patient survival rate of 50% (2,3). Elucidating the mechanisms underlying BC invasion and metastasis is indispensable for the development of effective therapies for this disease.

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The transforming growth factor-β (TGF-β) signaling pathway plays an important role in carcinoma development (4,5). This signaling pathway induces epithelial-mesenchymal transition (EMT) and promotes cell invasiveness and metastasis in multiple cancers, including BC (6-8). TGF-β binds to cell surface transmembrane serine/threonine kinase receptors and transduces signals principally through Smad proteins, which induce cellular responses by directly activating the expression of EMT transcription factors (9). Several mechanisms are involved in the regulation of TGF-β signaling, such as positive regulation by stimulatory factors and negative regulation by negative feedback mechanisms (10).

SnoN (also known as SKI-like proto-oncogene, SKIL), a member of the Ski family, is a negative regulator of TGF-β signaling (11). SnoN acts as a Smad corepressor in the nucleus by interacting with Smad complexes and recruiting other corepressors to inhibit Smad transcriptional activities (11). In the cytoplasm, SnoN blocks TGF-β signals by sequestering Smad proteins and preventing their translocation to the nucleus (12). TGF-β also tightly regulates SnoN levels in a biphasic manner: short stimulation with TGF-β causes rapid and transient SnoN protein degradation via the ubiquitin-proteasome system, whereas longer TGF-β treatment increases SnoN levels by inducing SnoN gene expression (13). SnoN thus participates in a negative feedback loop to regulate TGF-β signaling (14). Recent studies suggest that the ability of SnoN to repress TGF-β signaling is regulated by SUMOylation, a post-translational modification that is catalyzed by a small ubiquitin-like modifier (SUMO)-activating E1 enzyme, a SUMO-conjugating E2 enzyme, and a SUMO E3 ligase (15-17). Investigation of the role of SnoN in cancer revealed both pro-oncogenic and anti-oncogenic activities (18). However, the role of SnoN in BC remains to be elucidated.

In the present study, the expression of SnoN did not differ between BC tissues and adjacent normal tissues and between BC cell lines and normal cells, whereas its function in repressing TGF-β was significantly attenuated compared with that in the normal control. TIF1γ, a newly identified SUMO E3 ligase, promoted SnoN SUMOylation and restored the ability of SnoN to repress TGF-β signaling. The present study

demonstrated that the TIF1γ-SnoN1 pathway has an inhibitory effect on TGF-β-induced EMT and invasion in BC.

Materials and methods

Tissue samples and cell culture. A total of 33 bladder tumor tissues and matched adjacent normal tissues were collected from Qilu Hospital of Shandong University (Qingdao, China) between 2010 and 2013 with informed consent. All specimens were frozen in liquid nitrogen immediately and subsequently confirmed by pathological analysis. The study was approved by the Ethics Committee of Shandong University (Jinan, China).

The human bladder cancer cell lines T24 and TCCSUP and the normal urothelial epithelial cell line SV-HUC-1 were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were grown in complete growth medium at 37˚C with 5% CO_2, as recommended by the manufacturer.

Real-time PCR assay. Total RNA was extracted from tissues and cells using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse-transcribed using oligo(dT) primers and SuperScript II transcriptase (Invitrogen Life Technologies). The cDNAs were subjected to quantitative PCR using the following primers: SnoN forward 5'-CTCACAAAGACAGAGGCAAGTA-3' and reverse, 5'-CCTCAAGTGAGACATCTGGATAAG-3'; TIF1γ forward, 5'-CAGCTCCTGGTTATACTCCTAATG-3' and reverse 5'-GAGTCGAAGCTGTGCTAAGT-3'; and Power SYBR Green PCR Master Mix (Invitrogen Life Technologies) on an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Grand Island, NY, USA). β-actin was used as the reference gene and gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method (19).

Western blotting. Proteins were extracted from cultured cells using lysis buffer (Beyotime, Nantong, China) and then quantified with the bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein were resolved by 10% SDS-PAGE and then transferred to nitrocellulose membranes. After blocking with 5% non-fat milk, the membranes were incubated overnight with the following primary antibodies: mouse anti-SnoN, rabbit anti-TIF1γ (both from Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-E-cadherin, mouse anti-N-cadherin (both from Cell Signaling Technology, Danvers, MA, USA) and mouse anti-fibronectin (Santa Cruz Biotechnology). Subsequently, the membranes were next incubated with horseradish peroxidase-conjugated secondary antibodies and target proteins were detected using an enhanced chemiluminescence system (Pierce Biotechnology, Inc., Rockford, IL, USA).

p3TP-lux luciferase reporter assay. TGF-β-dependent transcriptional activation was detected with the p3TP-lux luciferase reporter, which consists of firefly luciferase under the control of three consecutive 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response elements (20). Cells were transiently transfected with the p3TP-lux reporter plasmid (Addgene, Cambridge, MA, USA) using FuGENE6 (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. The phRL-TK vector (Promega, Madison, WI, USA) was co-transfected to determine transfection efficiency. After 24 h, the cells were treated with or without TGF-β (Biolegend, San Diego, CA, USA) for the indicated times. Cells were then lysed and luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. p3TP-lux luciferase activity was normalized to that of the control phRL-TK vector.

Lentivirus-mediated overexpression and RNA interference. To construct the overexpression lentivirus plasmid, the coding DNA sequence of SnoN or TIF1γ was PCR amplified from cDNA of cultured normal epithelial cells, and cloned into the pHBLV-CMVIE-IRES-Puro lentiviral vector (Hanbio, Shanghai, China). The recombinant lentivirus (Lv-SnoN or Lv-TIF1γ) was produced by co-transfection of 293T cells with the plasmids psPAX2 and pMD2G using LipoFiter (Hanbio). To knock down SnoN, a lentivirus with a SnoN shRNA sequence (Lv-shSnoN) was purchase from Santa Cruz Biotechnology. The empty lentivector or control shRNA lentiviral particles (Santa Cruz Biotechnology) were used as the negative control (Lv-NC). Cells were exposed to the lentivirus-containing supernatant for 24 h in the presence of polybrene (Sigma-Aldrich, St. Louis, MO, USA). Stable transfectants were selected with puromycin (2 mg/ml) and verified by western blotting and real-time PCR.

Immunoprecipitation assay. Cells were lysed in TNTE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 1 mM EDTA) containing 0.5% Triton X-100 plus protease and phosphatase inhibitors. *N*-ethylmaleimide (NEM, 20 mM), an isopeptidase inhibitor, or vehicle alone was included in the lysis buffer where indicated. Cell lysates were centrifuged at 15,000 x g for 10 min at 4˚C and the supernatant was subjected to immunoprecipitation using mouse anti-SnoN antibody (Santa Cruz Biotechnology). Immunoprecipitated proteins were then separated by SDS-PAGE followed by immunoblotting using rabbit anti-SnoN and rabbit anti-SUMO antibodies (Santa Cruz Biotechnology), and visualized as described for western blotting.

Transwell invasion assay. Cell invasion was assessed using the Transwell chamber invasion assay. Cells ($1x10^5$) were added to the top chamber with Matrigel-coated membranes (8-μm pore size; Millipore, Bedford, MA, USA). Medium with 10% fetal bovine serum was added to the lower chamber as a chemoattractant. TGF-β1 (10 ng/ml) or vehicle alone was added to the upper and lower chambers. After 48 h, cells that had invaded to the lower surface of the membrane were stained with 0.1% crystal violet and counted in five random fields.

Statistical analyses. The data from independent experiments repeated at least three times are presented as the mean ± standard error of the mean (SEM). Statistical significance ($p<0.05$) was determined by the Student's t-test or analysis of variance followed by Bonferroni's post hoc tests.

Results

SnoN regulation of the TGF-β pathway is absent in BC. The expression levels of SnoN were examined in BC tissues and cell

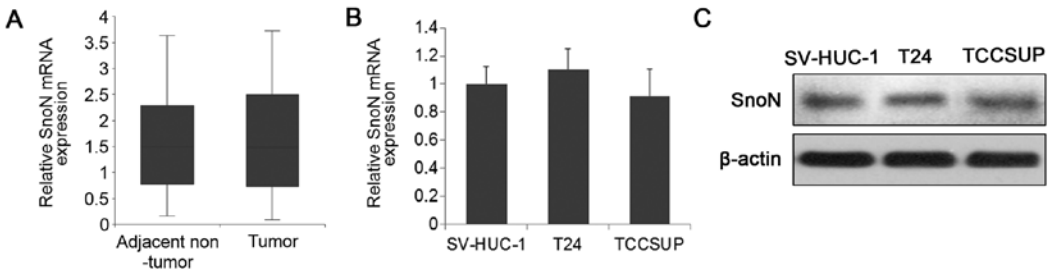

Figure 1. Expression of SnoN in bladder cancer (BC) tissues and cells. (A) SnoN expression in 33 pairs of BC (tumor) and surrounding tissues (adjacent non-tumor) was detected by real-time PCR. (B) Real-time PCR and (C) western blotting analyses of SnoN expression levels in normal urothelial epithelial cells (SV-HUC-1) and BC cells (T24 and TCCSUP).

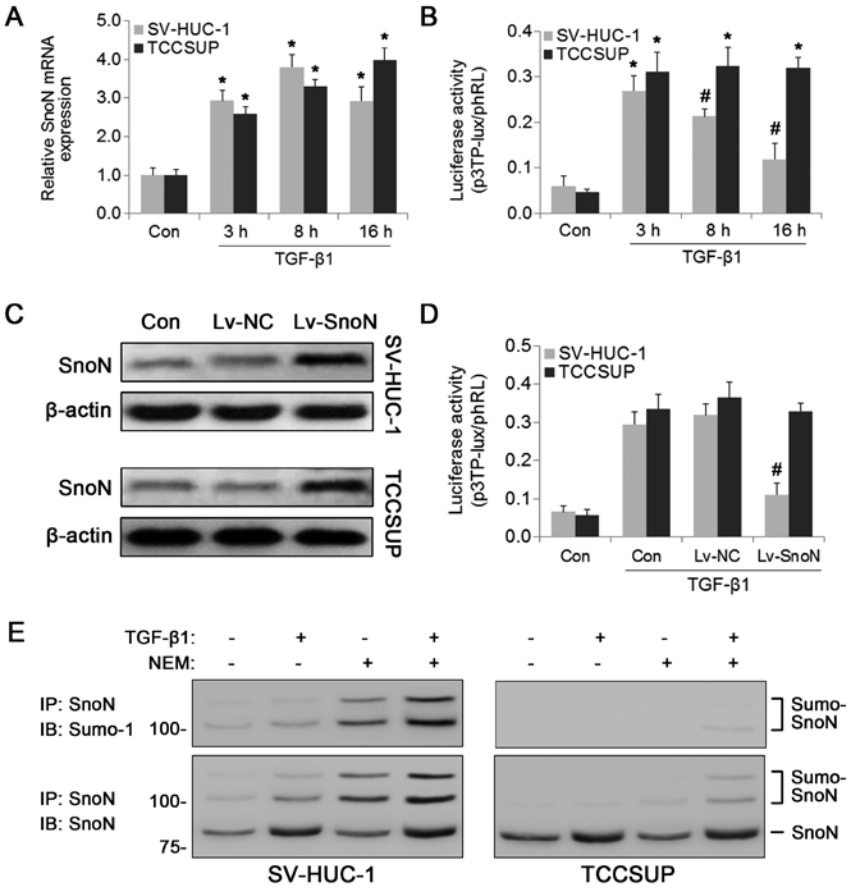

Figure 2. Negative regulation of the TGF-β pathway by SnoN is blocked in BC. (A) SV-HUC-1 and TCCSUP cells were incubated with or without 10 ng/ml TGF-β1 for the indicated times. SnoN expression levels were detected by real-time PCR. *P<0.05, compared with control. (B) SV-HUC-1 and TCCSUP cells were transiently transfected with plasmids containing p3TP-lux and phRL-TK sequences and incubated with or without TGF-β1 (10 ng/ml) for the indicated times. Cells were lysed and reporter activities were determined. *P<0.05, compared with control and #p<0.05, compared with TGF-β1 treatment for 3 h. (C) SV-HUC-1 and TCCSUP cells were stably transfected with Lv-NC or Lv-SnoN. SnoN expression levels were detected by western blotting. (D) SV-HUC-1 and TCCSUP cells stably transfected with Lv-SnoN were then transiently transfected with the p3TP-lux reporter and phRL-TK plasmid. After TGF-β1 (10 ng/ml) treatment for 3 h, cells were harvested and luciferase activity was analyzed. #P<0.05, compared with TGF-β1-treated control. (E) After incubation with or without TGF-β1 (10 ng/ml) for 8 h, SV-HUC-1 and TCCSUP cells were lysed in the presence or absence of the isopeptidase inhibitor NEM. Lysates were subjected to immunoprecipitation (IP) with anti-SnoN antibody and followed by immunoblotting (IB) with anti-Sumo-1 and anti-SnoN.

lines. Real-time PCR assessment of 33 BC and adjacent normal tissue samples showed that SnoN expression did not differ significantly between the BC and normal tissues (Fig. 1A). Similar results were obtained when comparing SnoN expression levels between BC cells and normal urothelial epithelial cells (Fig. 1B and C). Since SnoN is an important participant of a negative feedback loop regulating the TGF-β pathway, we next examined the effect of TGF-β on SnoN expression. Consistent with a previous study (13), TGF-β positively regulated SnoN mRNA expression in both normal and BC cells at least in the first 8 h (Fig. 2A). However, at 16 h after TGF-β treatment, SnoN expression began to decline in the SV-HUC-1 cells, whereas high SnoN expression levels were maintained in the TCCSUP cells. TGF-β-dependent transcriptional activation was examined next using the p3TP-lux luciferase reporter assay. As shown in Fig. 2B, the luciferase activities of

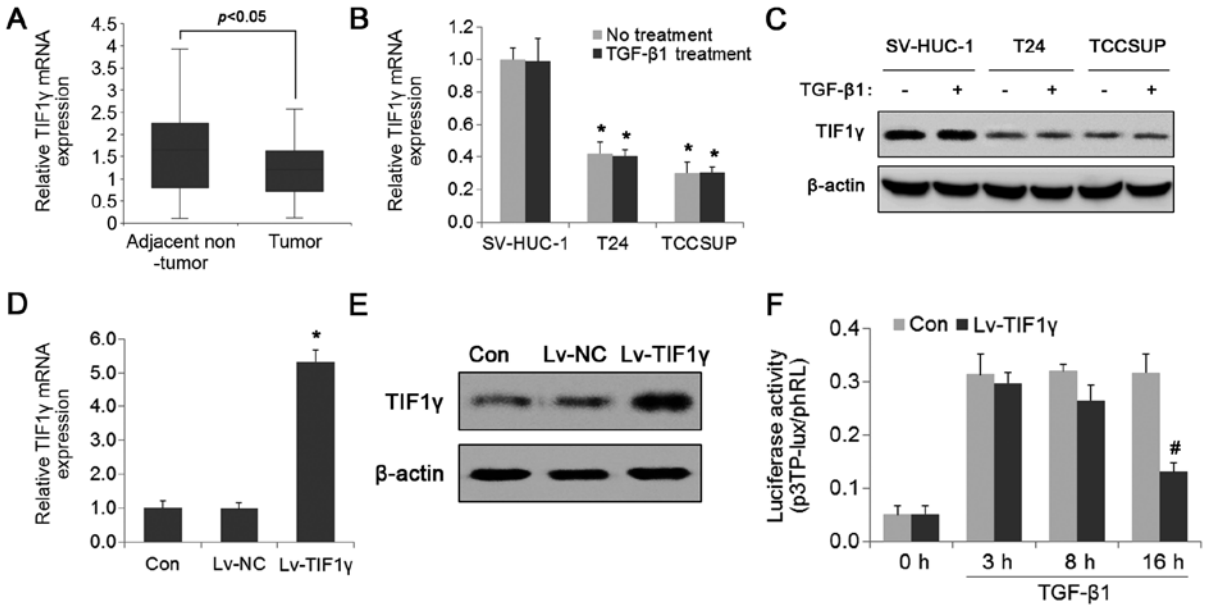

Figure 3. Expression of TIF1γ is downregulated in BC and its restoration represses the TGF-β pathway. (A) TIF1γ expression in 33 pairs of BC (tumor) and surrounding tissues (adjacent non-tumor) was detected by real-time PCR. SV-HUC-1, T24 and TCCSUP cells were incubated with or without TGF-β1 (10 ng/ml) for 16 h, TIF1γ expression levels were analyzed by (B) real-time PCR and (C) western blotting. *P<0.05, compared with SV-HUC-1 cells. SV-HUC-1 and TCCSUP cells were stably transfected with Lv-NC or Lv-TIF1γ, and SnoN expression levels were detected by (D) real-time PCR and (E) western blotting. *P<0.05, compared with control. (F) SV-HUC-1 and TCCSUP cells stably transfected with Lv-TIF1γ were then transiently transfected with the p3TP-lux reporter and phRL-TK plasmid. After TGF-β1 (10 ng/ml) incubation for the indicated times, luciferase activity was detected. #P<0.05, compared with control.

p3TP-lux were significantly increased in response to TGF-β treatment for 3 h in both cell lines; however, p3TP-lux activity was suppressed at 8 and 16 h compared with that at 3 h in the normal urothelial epithelial cell line SV-HUC-1, but not in the BC cells. These results suggest that the negative regulation of TGF-β signaling by SnoN was blocked in the BC cells. To confirm the role of SnoN in the regulation of the TGF-β pathway in BC cells, SnoN was overexpressed in TCCSUP and SV-HUC-1 cells using a lentiviral vector (Fig. 2C). As shown in Fig. 2D, TGF-β-dependent transcriptional activity was reduced by SnoN overexpression in the SV-HUC-1 cells but not in the TCCSUP cells. Previous studies indicated that post-translational modification by SUMOylation may contribute to the ability of SnoN to regulate transcription (16,21). Therefore, we examined the SUMOylation status of SnoN using immunoprecipitation assays. In the presence of the SUMO-protease inhibitor NEM, SUMOylated SnoN was detected in the SV-HUC-1 cells in the presence or absence of TGF-β treatment (Fig. 2E). However, the SUMO immunoreactive protein bands were undetectable in the TCCSUP cells in the absence of TGF-β treatment and detected at low levels in the presence of TGF-β (Fig. 2E). These results indicated that the regulatory function of SnoN in the TGF-β pathway was absent in BC cells, which could be attributed to the weak SUMOylation of SnoN.

Restoration of TIF1γ expression represses the TGF-β pathway in BC. Next, we investigated the mechanisms underlying the abnormal SUMOylation of SnoN in BC cells. TIF1γ is a member of multiple families (22) and was recently reported to function as a SUMO E3 ligase that promotes the SUMOylation of SnoN (15). Because TIF1γ-induced SUMOylation is required for SnoN to suppress TGF-β-induced EMT in mouse mammary epithelial cells (15), we examined whether TIF1γ affects SnoN SUMOylation and TGF-β signaling in BC cells. The expression levels of TIF1γ in BC tissues and cells were first evaluated. As shown in Fig. 3A, TIF1γ mRNA expression was significantly downregulated in the BC tissues compared with that noted in the adjacent normal control tissues. Similar results were obtained when comparing the mRNA and protein expression of TIF1γ between BC cells and normal epithelial cells (Fig. 3B and C). Unlike SnoN, TIF1γ expression was not significantly affected by TGF-β (Fig. 3B and C). To further assess the effect of TIF1γ on TGF-β signaling, TIF1γ was stably overexpressed in the TCCSUP cells by lentivirus and the expression levels were assessed by real-time PCR and western blotting (Fig. 3D and E). The p3TP-lux luciferase reporter assay showed that luciferase activity was significantly reduced in the TIF1γ-overexpressing TCCSUP cells after 16 h of TGF-β1 treatment compared with that at 3 h (Fig. 3F). A similar trend was observed in the SV-HUC-1 cells (Fig. 2B), suggesting that restoring TIF1γ recovered the negative regulation of the TGF-β pathway in BC cells.

SnoN is necessary for TIF1γ-mediated negative regulation of the TGF-β pathway in BC cells. To investigate whether the suppressive effect of TIF1γ on the TGF-β pathway is mediated by SnoN SUMOylation, SnoN expression was knocked down in the TIF1γ-overexpressing TCCSUP cells by lentiviral transient transfection. Real-time PCR and western blotting confirmed that the expression of SnoN was markedly reduced after lentiviral transfection (Lv-shSnoN) (Fig. 4A and B). Next, the effect of SnoN knockdown on TGF-β-dependent transcriptional activation was examined. As shown in Fig. 4C, SnoN silencing resulted in the recovery of p3TP-lux activity in the TIF1γ-overexpressing cells. Assessment of the effect

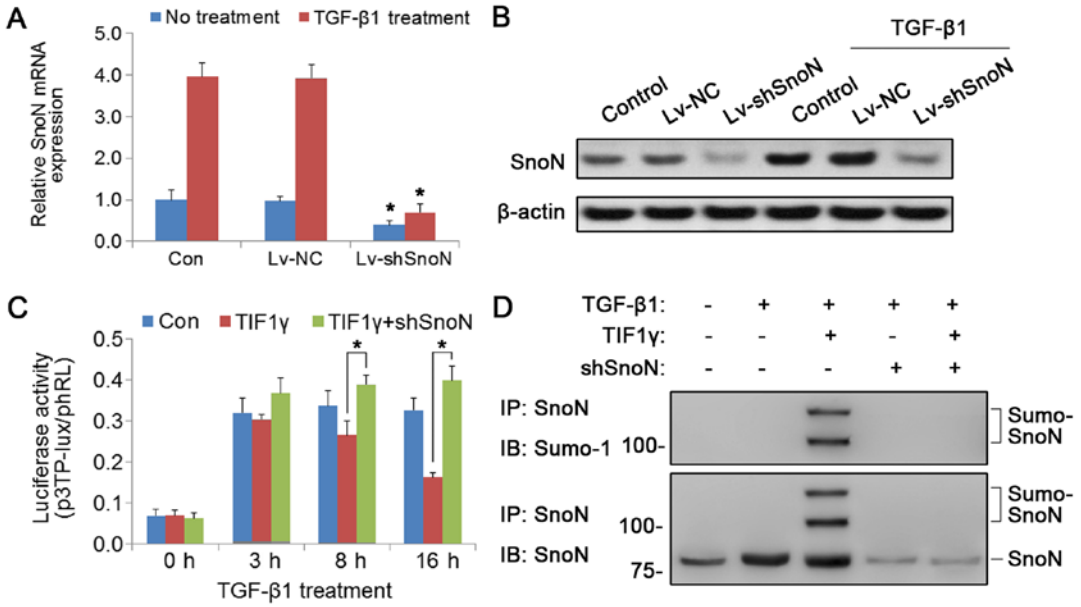

Figure 4. SnoN knockdown abrogates the TIF1γ-mediated suppression of the TGF-β pathway in BC cells. TCCSUP cells overexpressing TIF1γ were stably transfected with Lv-shSnoN and incubated with or without TGF-β1 (10 ng/ml) for 16 h. TIF1γ expression levels were analyzed by (A) real-time PCR and (B) western blotting. *P<0.05, compared with control. (C) Indicated cells were transfected with the p3TP-lux reporter and phRL-TK plasmid. After TGF-β1 (10 ng/ml) treatment for the indicated times, luciferase activity was detected. *P<0.05. (D) Indicated cells were incubated with or without TGF-β1 (10 ng/ml) for 8 h. Cells were lysed in the presence of the isopeptidase inhibitor NEM, and lysates were subjected to immunoprecipitation (IP) with anti-SnoN antibody followed by immunoblotting (IB) with anti-Sumo-1 and anti-SnoN.

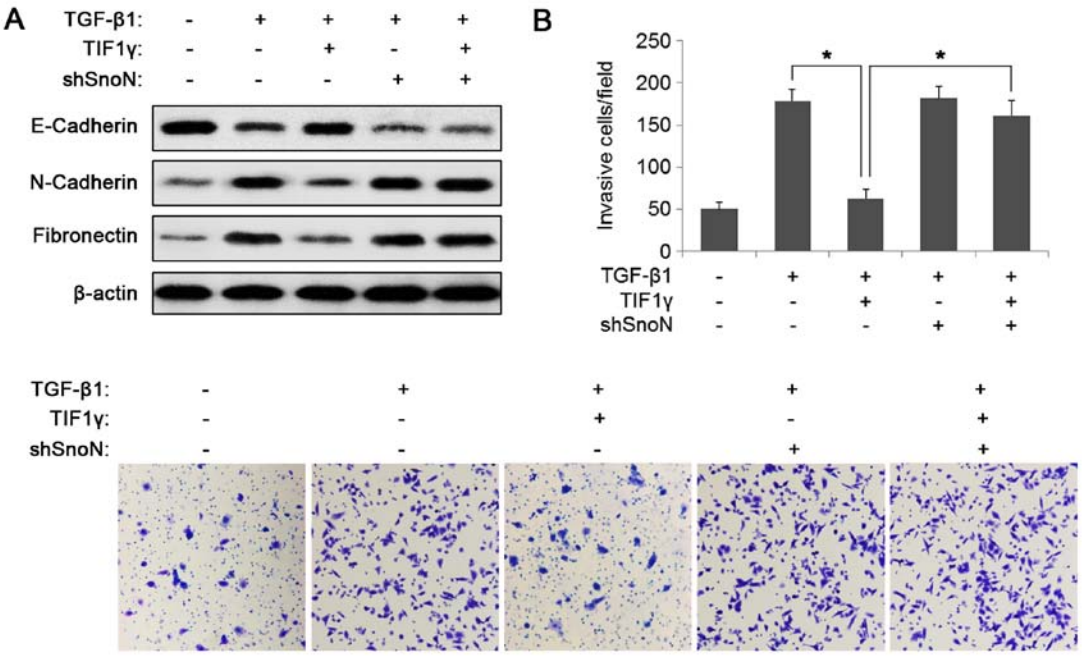

Figure 5. Effects of TIF1γ overexpression and SnoN knockdown on TGF-β-induced EMT and invasion in BC. Indicated cells were incubated with or without TGF-β1 (10 ng/ml) for 24 h. (A) The expression levels of E-cadherin, N-cadherin, and fibronectin were detected by western blotting. (B) The invasive ability of cells was detected by Transwell assay and representative images are shown (bottom panel). *P<0.05.

of TIF1γ on SnoN SUMOylation by immunoprecipitation showed that TIF1γ overexpression increased the levels of SUMOylated SnoN, and this effect was abrogated by SnoN knockdown (Fig. 4D). All things considered, these results indicated that TIF1γ promoted the SUMOylation of SnoN, which was necessary for the inhibitory effect of TIF1γ on the TGF-β pathway in BC cells.

TIF1γ restores the effect of SnoN on inhibiting TGF-β-induced EMT and invasion in BC. TGF-β induces EMT and promotes tumor metastasis in BC (7,23). To test whether the TIF1γ-SnoN SUMOylation pathway plays a role in TGF-β-induced EMT, the effects of TIF1γ overexpression and/or SnoN silencing on the expression of EMT markers were examined. As shown in Fig. 5A, TGF-β-induced changes of EMT markers

(decreased expression of E-cadherin and increased expression of N-cadherin and fibronectin) were attenuated by TIF1γ overexpression. Consistent with the SnoN-mediated suppression of TIF1γ on the TGF-β pathway, SnoN knockdown abrogated the effect of TIF1γ on TGF-β-induced EMT (Fig. 5A). The roles of TIF1γ and SnoN in TGF-β-induced cell invasion using the Transwell assay were then examined. As shown in Fig. 5B, TIF1γ overexpression significantly reduced TGF-β-induced cell invasion and knockdown of SnoN blocked this ability of TIF1γ. These data suggest that the inhibitory effects of TIF1γ on TGF-β-induced EMT and invasion are mediated by SnoN in BC. TIF1γ thus restored the function of SnoN as an inhibitor of TGF-β-induced EMT and invasion in BC.

Discussion

TGF-β signaling is an important pathway that regulates many cell functions and is implicated in diverse physiological and pathological events. To ensure its proper physiological function, TGF-β signaling is tightly regulated at different levels in different cells and tissues (24). Dysregulation of TGF-β signaling induces EMT and contributes to tumor progression (6). In the present study, we demonstrated that the loss of the regulatory function of SnoN in TGF-β signaling is a potential mechanism whereby TGF-β induces EMT and promotes tumor metastasis in BC.

SnoN can be induced by TGF-β1 and is a negative regulator of TGF-β1 signaling, which suggests that a negative feedback mechanism modulates TGF-β1 signaling (11). Alterations in SnoN expression in certain cancers are associated with tumorigenesis and the prognosis of patients (25-27). Our results showed no differences in SnoN expression between BC tissues or cells and adjacent normal tissues or normal urothelial epithelial cells. However, TGF-β induced the expression of SnoN for a longer period of time in BC cells than in normal epithelial cells. These results together with the findings that TGF-β-dependent transcriptional activity gradually declined from its peak in normal epithelial cells, but not in BC cells, suggest that SnoN is dysfunctional in BC cells. In line with this hypothesis, overexpressed SnoN had no effect on TGF-β signaling in BC cells.

Post-translational modifications regulate protein function, and SUMOylation is an important modification that affects SnoN activity (16). SUMOylation occurs via the covalent attachment of the protein SUMO to a lysine residue on a substrate, and this process is catalyzed by the sequential action of three sets of enzymes (17). Here, it was found that TIF1γ, a newly identified SUMO E3 ligase, was significantly downregulated in BC tissues and cells compared with normal controls. Restoring TIF1γ significantly repressed TGF-β signaling after a specific period, showing a similar trend to that in normal epithelial cells treated by TGF-β1. TIF1γ (also referred to as Trim33) is a member of the tripartite motif/RING finger, B-boxes, and a coiled-coil domain (TRIM/RBCC) family and E3 ubiquitin-ligase family (28). TIF1γ functions as a suppressor of the TGF-β superfamily signaling by inhibiting the formation of Smad nuclear complexes (29,30). Recently, TIF1γ was shown to induce the SUMOylation on SnoN by acting as a SUMO E3 ligase, and SUMOylation is required for SnoN mediated abrogation of TGF-β1 signaling (15). The results here showed that TIF1γ suppression of TGF-β1 signaling was dependent on SnoN expression in BC cells, suggesting that TIF1γ plays a role as a suppressor of TGF-β1 by restoring the regulatory function of SnoN in BC. TIF1γ may play either a tumor-suppressor or -promoter role in cancer (31,32). In BC cells, it was demonstrated that TIF1γ could inhibit TGF-β-induced EMT and invasion in the presence of normally expressed SnoN, implying that TIF1γ serves as a tumor suppressor in BC.

In summary, this study demonstrated that the loss of the function of SnoN as a suppressor of TGF-β resulted in the dysregulation of TGF-β signaling in BC. TIF1γ, as a SUMO E3 ligase, restored the function of SnoN, leading to the inhibition of TGF-β-induced EMT and invasion in BC.

Acknowledgements

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