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Overexpression of SASH1 Inhibits TGF- β 1-Induced EMT in Gastric Cancer Cells

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The epithelial–mesenchymal transition (EMT) is considered to be one of the critical steps in gastric cancer cell invasion and metastasis. SAM- and SH3-domain containing 1 (SASH1), a member of the SLY family of signal adapter proteins, is a candidate for tumor suppression in several cancers. However, the biological role of SASH1 in gastric cancer remains largely unknown. Therefore, the purpose of this study was to investigate the impact of SASH1 on the biological behavior of gastric cancer cells treated with transforming growth factor (TGF)- β 1. In the current study, we provide evidence that SASH1 was lowly expressed in human gastric cancer cells, and TGF- β 1 also inhibited the expression of SASH1 in TSGH cells. We found that SASH1 inhibited TGF- β 1-mediated EMT in TSGH cells, as well as cell migration and invasion. Furthermore, SASH1 obviously inhibited the phosphorylation of PI3K and Akt in TGF- β 1-stimulated TSGH cells. In summary, our study is the first to show that overexpression of SASH1 inhibits TGF- β 1-induced EMT in gastric cancer cells through the PI3K/Akt signaling pathway. These results suggest that SASH1 may be a potential therapeutic target for the treatment of gastric cancer.

Key words: SAM- and SH3-domain containing 1 (SASH1); Gastric cancer;
Epithelial–mesenchymal transition (EMT)

INTRODUCTION

Gastric cancer is the fourth most prevalent cancer type and the second largest contributor to cancer-related deaths in the world (1). Although the range of therapeutic strategies available for treatment of this cancer has improved in the past two decades, the 5-year survival rate of patients with gastric cancer is less than 20% because of the distant metastases and local recurrence (2). Therefore, the mechanisms of the biologic processes that drive metastasis need to be elucidated.

Epithelial–mesenchymal transition (EMT) is a crucial step in the initiation of metastatic spread of many tumor cells into distal organs (3). During EMT, epithelial cells lose their characteristic marker E-cadherin and gain mesenchymal markers, including N-cadherin and vimentin (4). Increasing evidence has reported that growth factors, including transforming growth factor (TGF)- β 1, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF), induce morphological cellular changes consistent with the acquisition of the EMT phenotype as characterized by the loss of epithelial

marker expression and the gain or increased expression of mesenchymal markers (5–8). Thus, suppressing the TGF- β 1 signaling pathway-mediated EMT might yield beneficial effects in treating cancer patients with advanced metastasis.

SAM- and SH3-domain containing 1 (SASH1), a member of the SLY family of signal adapter proteins, is a candidate for tumor suppression in several cancers. Reduced expression of SASH1 is associated with aggressive tumor growth, metastasis formation, and poor patient survival (9–12). However, the biological role of SASH1 in gastric cancer remains largely unknown. Therefore, the purpose of this study was to investigate the impact of SASH1 on the biological behavior of gastric cancer cells treated with TGF- β 1. We found that SASH1 is implicated in the regulation of TGF- β 1-induced EMT via the PI3K/Akt signaling pathway.

MATERIALS AND METHODS

Cell Culture

Human gastric cancer cell lines (AGS, MKN45, and TSGH) and a normal gastric mucosal epithelial cell line

(GES-1) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco Laboratories) at 37°C in a humidified incubator in 5% (v/v) CO₂ atmosphere.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was isolated from gastric cancer cells using the RNA plus kit (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA from each sample was subject to cDNA synthesis, using the Transcriptor First Strand cDNA Synthesis Kit (Invitrogen). PCR amplification was performed using 1.25 U Ex Taq polymerase (Takara Bio, Inc., Dalian, China). The primer sequences used for RT-PCR were as follows: SASH1 sense 5'-TCCCGTCAC AGGAAGAAACG-3' and antisense 5'-GATACCCATC ACGTCGGTCC-3'; β -actin sense 5'-CATCCGTAAAG ACCTCTATGCCAAC-3' and antisense 5'-ATGGAGCC ACCGATCCACA-3'. The primers were all synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Quantitative PCR thermal cycling program: 10 min at 95°C, 35 thermal cycles of 20 s at 95°C, 30 s at 59°C, and 30 s at 72°C. β -Actin was used as a control for normalizing gene expression. Relative gene expression was determined based on the threshold cycles (Ct) of the gene of interest and of the internal reference gene.

Western Blot

Total protein extracts were lysed in lysis buffer [20 mM HEPES (pH 7.6), 350 mM NaCl, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 50 mM NaF, 0.1 mM DTT, 0.1 mM PMSF, and a protease inhibitor cocktail]. The protein concentration was determined using a Bradford protein assay (Takara Biotechnology, Dalian, China). Equal amounts of protein were electrophoresed on SDS-PAGE and blotted onto PVDF membranes (Millipore Corp., Billerica, MA, USA). The membranes were blocked in 5% nonfat dry milk for 2 h and incubated with various primary antibodies (anti-SASH1, anti-E-cadherin, anti-N-cadherin, anti-phospho-PI3K, anti-PI3K, anti-phospho-Akt, anti-Akt or anti- β -actin; from Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, followed by horseradish peroxidase (HRP)-conjugated IgG (Santa Cruz Biotechnology). The immunoreactive protein bands were visualized by ECL kit (Pierce, Rockford, IL, USA).

Construction of the pcDNA3.1-SASH1 Vector and Cell Transfection

The full-length SASH1 open reading frame was amplified from TSGH cells by RT-PCR, and cloned into the

pcDNA3.1 expression vector to construct the pcDNA3.1-SASH1 recombinant expression vector. TSGH cells were transfected with pcDNA3.1-SASH1 or pcDNA3.1 (empty vector) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. After 48 h of transfection, the transfectants were selected in a medium containing 0.5 mg/ml of G418 for 2 to 3 weeks to generate the stable pools.

Cell Migration and Invasion Assays

TSGH cells were infected with pcDNA3.1-SASH1 or pcDNA3.1. After 24 h, cells were trypsinized and 3×10^4 cells were added to 8- μ m pore size inserts (BD Biosciences, Bedford, MA, USA) to perform transwell migration assay as per manufacturer's instructions. For invasion assays, cells were treated as described, but 3×10^4 cells were placed in 8- μ m pore size Matrigel-coated invasion chamber inserts (BD Biosciences) and incubated for 24 h. The lower chamber of the transwell was filled with 500 μ l DMEM containing 10% FBS as a chemoattractant. After 24 h of incubation at 37°C with 5% CO₂, the cells on the surface of upper chamber were removed by scraping with a cotton swab. The migrated/invaded cells on the lower surface of the filter were washed, fixed, stained with 0.1% crystal violet, and counted under a microscope.

Statistical Analysis

Data are expressed as mean \pm standard deviation (SD) and analyzed by one-way analysis of variance, Student's *t*-test or ANOVA. The difference between means was considered significant when the value was $p < 0.05$.

RESULTS

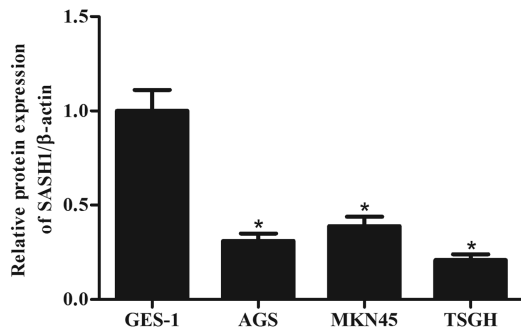
Expression of SASH1 Was Downregulated in Gastric Cancer Cell Lines

The expression of SASH1 gene was analyzed by RT-qPCR in human gastric cancer cell lines. As shown in Figure 1A, the expression of SASH1 mRNA in gastric cancer cell lines (AGS, MKN45, and TSGH) was obviously downregulated, respectively, compared to that of normal gastric mucosal epithelial cell line (GES-1). Western blot analysis also demonstrated that the expression of SASH1 protein was also significantly decreased in gastric cancer cell lines (AGS, MKN45, and TSGH) (Fig. 1B).

TGF- β 1 Inhibits the Expression of SASH1 in TSGH Cells

We investigated the response of SASH1 to TGF- β 1 in TSGH cells. TSGH cells were treated with 5 ng/ml TGF- β 1 for 0, 3, 6, 12, or 24 h, and the expression of SASH1 was detected by RT-qPCR and Western blot. As shown in Figure 2A, the expression of SASH1 mRNA was downregulated in response to TGF- β 1 in a time-dependent manner.

A



B

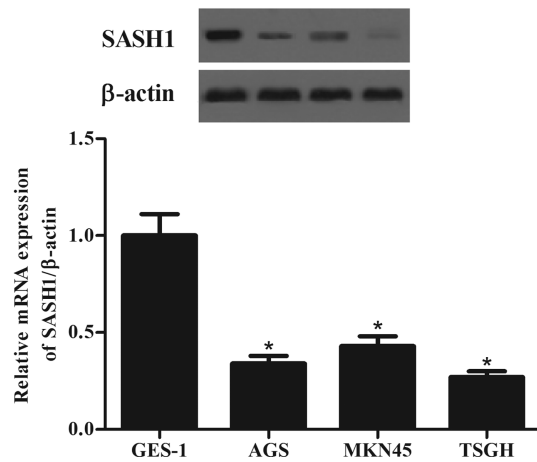


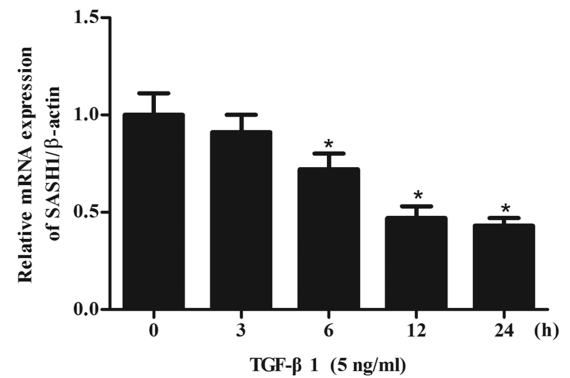
Figure 1. Expression of SASH1 was downregulated in gastric cancer cell lines. (A) The expression of *SASH1* gene was analyzed by RT-qPCR in human gastric cancer cell lines; β-actin served as the loading control. (B) The expression of SASH1 protein was detected by Western blot in human gastric cancer cell lines; β-actin served as the loading control. All experiments were repeated at least three times. The values shown represent the mean ± SD. * $p < 0.05$ versus the GES-1 group.

Similarly, TGF-β1 treatment significantly decreased the expression of SASH1 protein in a time-dependent manner (Fig. 2B).

SASH1 Inhibits TGF-β1-Mediated EMT in TSGH Cells

To examine whether SASH1 was involved in the TGF-β1-induced EMT, we measured the effects of SASH1 on EMT-related molecule expression in TSGH cells. The cells were infected with pcDNA3.1-SASH1 or pcDNA3.1. pcDNA3.1-SASH1 efficiently increased the protein expression of SASH1 (Fig. 3A). As shown in Figure 3B, TGF-β1 treatment significantly inhibited the expression of E-cadherin and promoted the protein expression of N-cadherin. However, the TGF-β1-induced

A



B

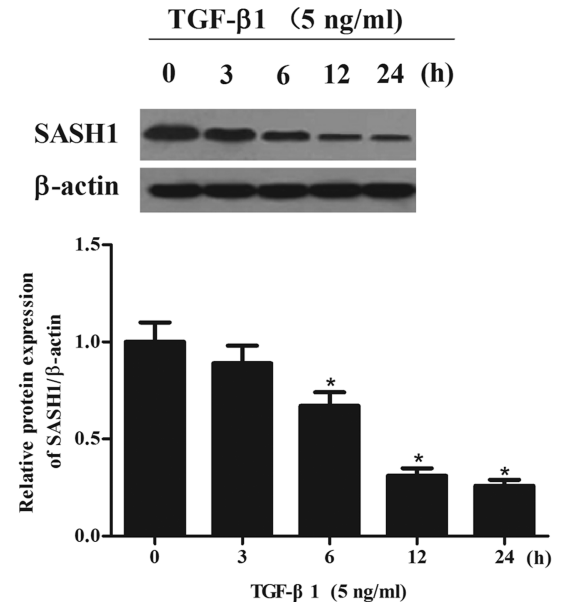


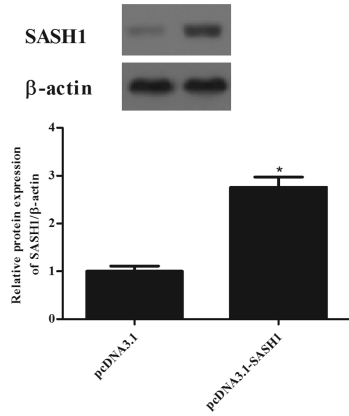
Figure 2. TGF-β1 inhibits the expression of SASH1 in TSGH cells. TSGH cells were treated with 5 ng/ml TGF-β1 for 0, 3, 6, 12, or 24 h, and the expression of SASH1 was detected by (A) RT-qPCR and (B) Western blot; β-actin served as the loading control. All experiments were repeated at least three times. The values shown represent the mean ± SD. * $p < 0.05$ versus the control group.

E-cadherin downregulation and N-cadherin upregulation were reversed in TSGH cells transfected with SASH1.

SASH1 Inhibits TGF-β1-Induced Migration and Invasion in TSGH Cells

We explored the effect of SASH1 on gastric cancer cell migration and invasion under TGF-β1 conditions. Our results showed that SASH1 overexpression greatly

A



B

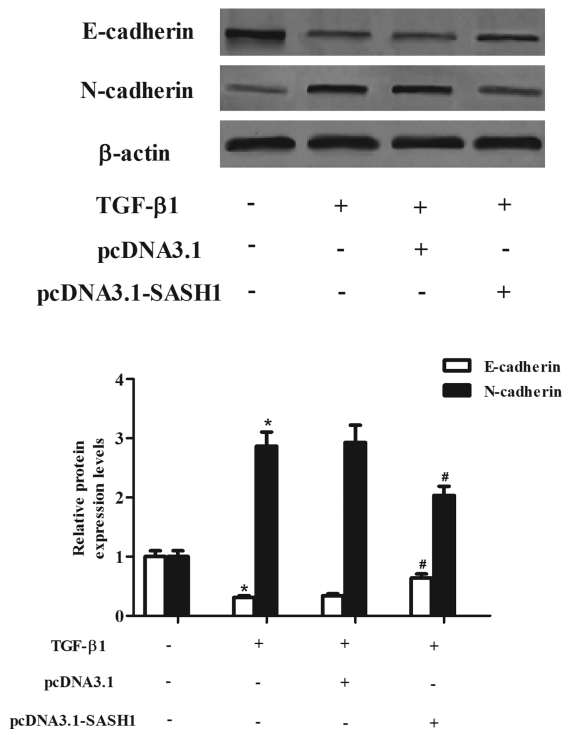
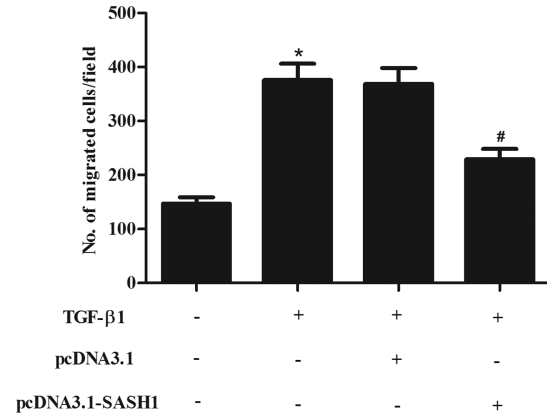


Figure 3. SASH1 inhibits TGF- β 1-mediated EMT in TSGH cells. TSGH cells transfected with the pcDNA3.1-SASH1 or pcDNA3.1 were treated with TGF- β 1 (5 ng/ml) for 24 h. The E-cadherin and N-cadherin protein expression levels were analyzed via Western blot, and representative blots are shown. Quantification analysis was performed using Gel-Pro Analyzer version 4.0 software. All experiments were repeated at least three times. The values shown represent the mean \pm SD. * p < 0.05 versus the control group. # p < 0.05 versus the pcDNA3.1 + TGF- β 1 group.

inhibited migration of TSGH cells induced by TGF- β 1 as assessed by transwell migration assay (Fig. 4A). SASH1 overexpression also significantly prevented TGF- β 1-induced cell invasion through Matrigel-coated polycarbonate filter in the transwell chamber (Fig. 4B).

A



B

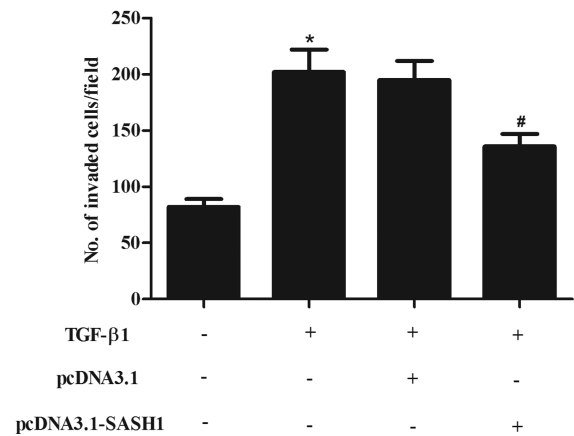


Figure 4. SASH1 inhibits TGF- β 1-induced migration and invasion in TSGH cells. Cells transfected with the pcDNA3.1-SASH1 or pcDNA3.1 were treated with TGF- β 1 (5 ng/ml) for 24 h. (A) Cell migration was measured by the transwell migration assay. (B) The invasive capability was determined using Matrigel invasion chambers. All experiments were repeated at least three times. The values shown represent the mean \pm SD. * p < 0.05 versus the control group. # p < 0.05 versus the pcDNA3.1 + TGF- β 1 group.

SASH1 Inhibits the Phosphorylation of PI3K and Akt in TGF- β 1-Stimulated TSGH Cells

Recent studies have suggested that activation of PI3K/Akt signaling induces the EMT process (13–15). Thus, we explored the role of the PI3K/Akt signaling in SASH1-mediated EMT in gastric cancer cells. As indicated in Figure 5, compared with the control group, TGF- β 1 treatment significantly increased the expression of p-PI3K

and p-Akt. However, SASH1 obviously inhibited the phosphorylation of PI3K and Akt in TGF- β 1-stimulated TSGH cells.

DISCUSSION

In the current study, we provide evidence that SASH1 was lowly expressed in human gastric cancer cells, and TGF- β 1 also inhibited the expression of SASH1 in TSGH cells. We found that SASH1 inhibits TGF- β 1-mediated EMT in TSGH cells, as well as cell migration and invasion. Furthermore, SASH1 obviously inhibited the phosphorylation of PI3K and Akt in TGF- β 1-stimulated TSGH cells.

Emerging studies have indicated that SASH1 is a candidate for tumor suppression in several cancers. Meng et al. confirmed that the expression of SASH1 in osteosarcoma tissues was significantly lower than in normal bone tissue, and the expression rate of SASH1 mRNA in the carcinoma tissues from patients with lung metastasis was significantly lower than that from patients without lung metastasis (12). In breast cancer, SASH1 expression was obviously reduced in breast cancer tissue compared with adjacent normal tissues, and its expression was regulated by methylation (16). Consistent with these findings, we demonstrated that SASH1 was lowly expressed in human gastric cancer cells, and TGF- β 1 also inhibited the expression of SASH1 in TSGH cells. These results suggest that SASH1 acts as a tumor suppressor whose downregulation may contribute to the progression and metastasis of gastric cancer.

Increasing evidence has reported that EMT plays an important role in tumor invasion and metastasis from in vitro and in vivo gastric cancer studies (17–19). E-cadherin plays a pivotal role in epithelial cell–cell adhesion, and the loss of E-cadherin is considered a hallmark of EMT (20). In addition, several studies demonstrated that stimulation of TGF- β 1 could induce EMT in gastric cancer (21–23). In this study, we observed that TGF- β 1 treatment significantly inhibited the expression of E-cadherin and promoted the expression of N-cadherin, whereas SASH1 inhibits TGF- β 1-mediated EMT in TSGH cells. Interestingly, several studies have documented that SASH1 plays an important role in promoting tumor invasion. Chen et al. reported that overexpression of the SASH1 significantly decreased migration of A549 human lung cancer cells (24). Yang et al. demonstrated that overexpression of the SASH1 also inhibited glioma cell invasion (25). In line with these previous studies, we found that SASH1 overexpression significantly prevented TGF- β 1-induced gastric cancer migration and invasion. These results suggest that SASH1-mediated inhibition of EMT may inhibit the migration and invasion in TGF- β 1-stimulated gastric cancer cells.

A number of mechanisms relevant to EMT initiation have been documented in the development of gastric

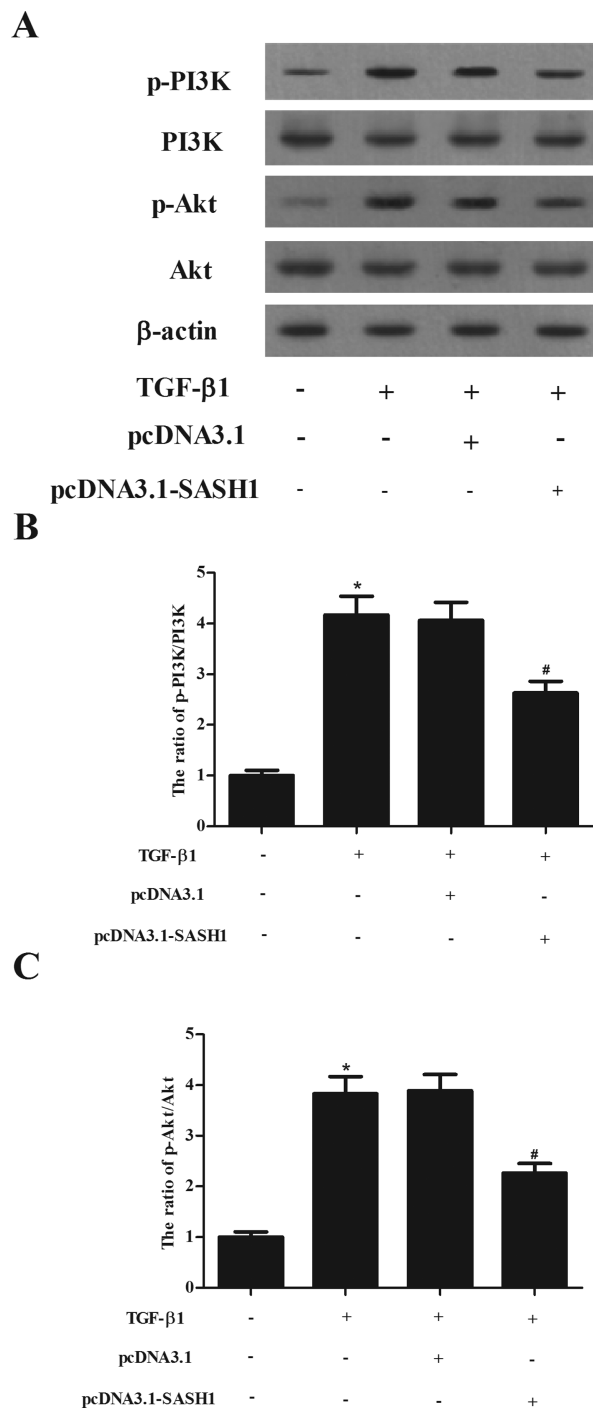


Figure 5. SASH1 inhibits the phosphorylation of PI3K and Akt in TGF- β 1-stimulated TSGH cells. TSGH cells transfected with the pcDNA3.1-SASH1 or pcDNA3.1 were treated with TGF- β 1 (5 ng/ml) for 30 min. The expression of p-PI3K, PI3K, p-Akt, and Akt proteins was detected by Western blot, and representative blots are shown. Quantification analysis was performed using Gel-Pro Analyzer version 4.0 software. All experiments were repeated at least three times. The values shown represent the mean \pm SD. * p < 0.05 versus the control group. # p < 0.05 versus the pcDNA3.1 + TGF- β 1 group.

cancer, including the Wnt/ β -catenin, TLR4/NF- κ B, and PI3K/Akt pathways (26–28). Akt, a serine/threonine protein kinase, was activated through the PI3K pathway, which has been implicated in cancer cell migration, invasion, and EMT phenotype (13). In addition, previous reports have indicated that the activation of the PI3K/Akt pathway was induced by TGF- β 1 (29). Consistent with the previous studies, we observed that TGF- β 1 treatment significantly increased the expression of p-PI3K and p-Akt, whereas SASH1 obviously inhibited the phosphorylation of PI3K and Akt in TGF- β 1-stimulated TSGH cells. These results suggest that overexpression of SASH1 inhibits TGF- β 1-induced EMT in gastric cancer cells through the PI3K/Akt signaling pathway.

In summary, our study is the first to show that overexpression of SASH1 inhibits TGF- β 1-induced EMT in gastric cancer cells through the PI3K/Akt signaling pathway. These results suggest that SASH1 may be a potential therapeutic target for the treatment of gastric cancer.

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