Diallyl disulfide inhibits TGF-β1-induced upregulation of Rac1 and β-catenin in epithelial-mesenchymal transition and tumor growth of gastric cancer

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Abstract. Transforming growth factor-β1 (TGF-β1) has been demonstrated to promote epithelial-mesenchymal transition (EMT), invasion and proliferation in tumors via the activation of Rac1 and β-catenin signaling pathways. The present study investigated the effects of diallyl disulfide (DADS) on TGF-β1-induced EMT, invasion and growth of gastric cancer cells. TGF-β1 treatment augmented EMT and invasion, concomitantly with increased expression of TGF-β1, Rac1 and β-catenin in gastric cancer cells. DADS downregulated the expression levels of TGF-β1, Rac1 and β-catenin. DADS, TGF-β1 receptor inhibitor as well as Rac1 inhibitor antagonized the upregulation of the TGF-β1-induced expression of these genes, abolishing the enhanced effects of TGF-β1 on EMT and invasion. Blocking the TGF-β1 receptor through inhibition resulted in the decreased expression of Rac1 and β-catenin. Rac1 inhibitor reduced the TGF-β1-induced β-catenin expression. In addition, DADS and the aforementioned inhibitors attenuated the TGF-β1-induced tumor growth and the expression changes of E-cadherin, vimentin, Ki-67 and CD34 in nude mice. These data indicated that the blockage of TGF-β1/Rac1 signaling by DADS may be responsible for the suppression of EMT, invasion and tumor growth in gastric cancer.

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Abbreviations: TGF-β1, transforming growth factor-β1; DADS, diallyl disulfide; EMT, epithelial-mesenchymal transition; LIMK1, LIM kinase-1; PAK1, P21 activated kinase-1; ROCK1, Rho-associated, coiled-coil containing protein kinase 1; uPAR, urokinase-type plasminogen activator receptor; ERK, extracellular regulated protein kinases; MMP9, matrix metalloproteinase-9; PI3K, phosphoinositide 3-kinase; AKT, PKB, protein kinase B; JNK, c-Jun N-terminal kinase; RT-PCR, reverse transcription-polymerase chain reaction; CSE, cigarette smoke extract; HRP, horseradish peroxidase

Key words: diallyl disulfide, TGF-β1, Rac1, β-catenin, gastric cancer cell epithelial-mesenchymal transition, invasion

Introduction

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related deaths worldwide (1). An estimated 951,600 new gastric cancer cases and 723,100 gastric cancer-related deaths occurred worldwide in 2012 (2). The patients are usually diagnosed at an advanced stage with metastases and more than half of radically resected gastric cancer patients relapse, either locally or with distant metastases. Therefore, the 5-year survival is less than 10% and the prognosis of patients remains poor (1).

Diallyl disulfide (DADS), one of the sulfur compounds derived from garlic, exhibits anticancer activity by modulating signaling molecules in various pathways, indicating that DADS could be used as a potential therapeutic agent for the treatment or prevention of cancer (3).

Transforming growth factor-β (TGF-β) plays a pivotal role in cancer progression and metastasis by inducing epithelial-mesenchymal transition (EMT), in which cancer cells acquire the capability of motility and invasion (4). TGF-β induces EMT not only through the Smad-mediated gene expression regulation, but also by activating non-Smad signaling, such as PI3K/Akt, ERK, JNK, p38, Src tyrosine kinase and Rho GTPases pathways (4,5).

TGF-β1, one of the members of the transforming growth factor family, induces EMT via the downregulation of the expression of E-cadherin and the upregulation of the expression of vimentin in gastric cancer cells (6). Crosstalk between the TGF-β1/Smad and other pathways is critical during the development of TGF-β1-induced EMT. Wnt/β-catenin pathway has been demonstrated to mediate EMT. Apart from Wnt-dependent β-catenin transactivation, TGF-β1 regulates β-catenin nuclear translocation through a Smad-dependent manner (7). In addition, TGF-β1-induced EMT is mediated by ERK-dependent β-catenin upregulation and nuclear translocation in renal tubular epithelial cells (7). The β-catenin inhibitor can reverse TGF-β1-induced EMT in human airway epithelial cells (8). In addition, DADS inhibits the activation of the β-catenin pathway and EMT in breast cancer cells (9).

Activation of Rac1 GTPase/Pak1 pathway is involved in TGF-β1-induced EMT in prostate cancer cells (10). We have demonstrated that DADS restrained EMT, migration and invasion through the downregulation of Rac1-Pak1/Rock1-LIMK1 (11) and uPAR-ERK-Fra-1 (12) pathways in gastric cancer cells and inhibited the Wnt-1/β-catenin pathway through the upregulation of miR-200b and miR-22 in gastric cancer cells (13).

We proposed that DADS has inhibitory effects on TGF-β1-induced EMT and invasion, which may be associated with the downregulation of Rac1 and β-catenin in gastric cancer cells.

In the present study, we verified that TGF-β1 upregulated Rac1 and β-catenin in gastric cancer cells and that Rac1 regulated the expression of β-catenin. DADS treatment inhibited the expression of TGF-β1, resulting in the downregulation of Rac1 and β-catenin. The underlying mechanisms of DADS suppressive effects on TGF-β1-induced EMT, invasion and growth of gastric cancer were investigated.

Materials and methods

Cell culture and cell line establishment. Human gastric cancer MGC803 cell line was obtained from the Cancer Research Institute, Xiangya Medical College, Central South University in China. Cells were cultured in RPMI-1640 medium (Gibco; Life Technologies, Vienna, Austria) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Vienna, Austria) with the addition of 100 U/ml penicillin, 100 U/ml streptomycin and maintained at 37˚C in a humidified atmosphere of containing 5% CO_2. To establish TGF-β1-overexpressing cell lines, the plasmid pCMV6 containing the full-length cDNA of human TGF-β1 (pCMV6-TGF-β1) was constructed by OriGene Technologies (Rockville, MD, USA). MGC803 cells were transfected with TGF-β1-expressing plasmid and the control vector (pCMV6-Neo) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. The stable transfectants were established after G418 (Invitrogen; Thermo Fisher Scientific, Inc.) selection. The expression levels of TGF-β1 in stable cell lines were evaluated by western blot analysis.

Reagents and antibodies. Diallyl disulfide (DADS), purchased from Fluka Co. (Milwaukee, WI, USA), was dissolved in Tween-80 and stored at -20˚C after a 100-fold dilution with saline. Human recombinant TGF-β1 protein was purchased from R&D Systems (Minneapolis, MN, USA). The primary antibodies against TGF-β1 (cat. no. ab92486), Rac1 (cat. no. ab33186), β-catenin (cat. no. ab16051), Ki-67 (cat. no. ab66155), CD34 (cat. no. ab81289) and FITC-conjugated anti-mouse (cat. no. ab6785) or anti-rabbit (cat. no. ab6717) secondary antibodies were provided by Abcam (Cambridge, MA, UK). The primary antibodies against E-cadherin (cat. no. 24E10) and vimentin (cat. no. D21H3) were obtained from Cell Signaling Technology (Danvers, MA, USA). The mouse monoclonal against β-actin antibody (cat. no. sc-8432) and horseradish peroxidase (HRP)-conjugated secondary antibodies (cat. no. sc-2004 and cat. no. sc-2005) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TGF-β1 receptor inhibitor SB431542 and Rac1 inhibitor NSC23766 were obtained from Cayman Chemical (Ann Arbor, MI, USA). A total of 5 ng/ml TGF-β1, 30 mg/l DADS, 10 μmol/l SB431542 and 50 μmol/l NSC23766 were used for experiments *in vitro*.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from the cells using TRIzol reagent (Gibco-BRL; Thermo Fisher Scientific, Inc., Grand Island, USA). Reverse transcription was carried out using the RT-PCR system (Promega, Madison, WI, USA). PCR analysis was performed using the GeneAmp PCR kit (Promega). Primer sequences were as follows: TGF-β1 forward, TCTCCAGGCATTTCC ACTATTC and reverse, CTCAGGCATTCGTCAACATCTA; Rac1 forward, TGCCTGCTGTTGTAAATGTCTC and reverse, AAAGTTCAGTGCTCGGTGTTCT; β-catenin forward, GGA AGGGACAGTATCGTTTGTT and reverse, GCCTCAGCA TCTACCAGCATAG; E-cadherin forward, CTCCCAATACAT CTCCCTTCAC and reverse, CGCCTCCTTCTTCATCATAGT AA; vimentin forward, GCGAGGAGAGCAGGATTTCT and reverse, TCTTGTAGGAGTGTCGGTTGTT; β-actin forward, CTGGGACGACATGGAGAAAA and reverse, AAGGAA GGATGGAAGAGTGC. The PCR products were analyzed on 2% agarose gel containing ethidium bromide. Densitometric quantitation of products was determined using the Labwork analysis software (Labworks LLC, Lehi, UT, USA). The relative abundance was expressed as the ratio of the object gene to β-actin.

Western blot analysis. For total protein extraction, cells were lysed directly on ice for 30 min in lysis buffer [10 mmol/l Tris-HCl (pH 7.6), 100 mmol/l NaCl, 1 mmol/l EDTA (pH 8.0), 100 μg/ml PMSF and 1 μg/ml aprotinin]. The cell lysates were centrifugated at 12,000 rpm for 10 min and the supernatants were collected. Then protein contents were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA).

Protein extracts were loaded on a 10% SDS-polyacrylamide gel for electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membrane. The blots were blocked in 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 2 h at room temperature, and then incubated with primary antibodies (1:200-500) at 4˚C overnight. The membranes were washed in TBS-T and then incubated with HRP-conjugated secondary antibodies (1:1,000-1:2,000). After washed with TBS-T, the membranes were developed by an enhanced chemiluminescence plus (ECL Plus) kit

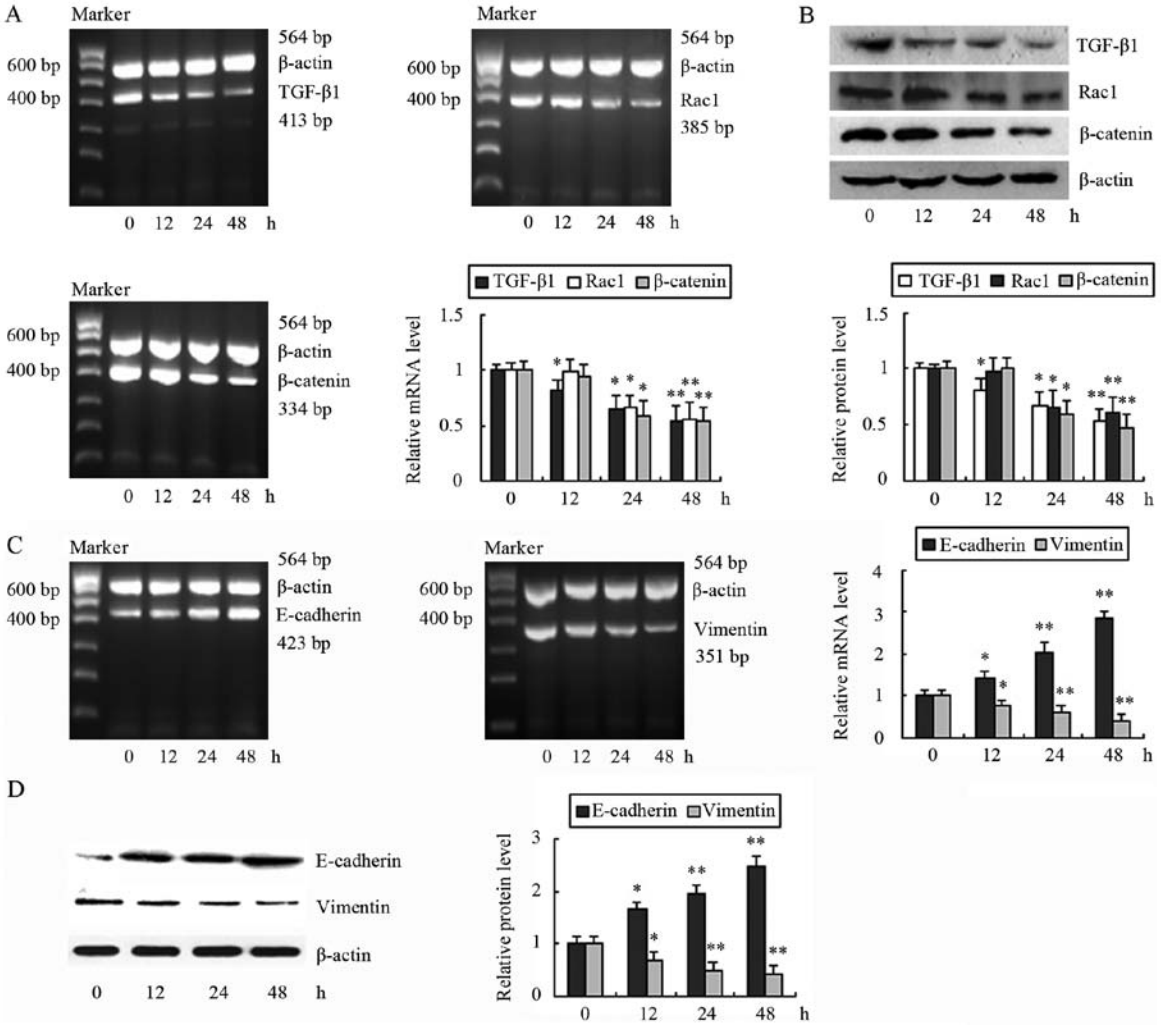

Figure 1. Analysis of the effects of DADS on the expression of TGF-β1, Rac1, β-catenin, E-cadherin and vimentin in MGC803 cells. (A and C) Cells were treated with 30 mg/l of DADS for the indicated time-points. RT-PCR was performed to detect the mRNA levels of (A) TGF-β1, Rac1, β-catenin, (C) E-cadherin and vimentin. β-actin was used as an internal control for normalization. (B and D) Western blot analysis was performed to detect the protein levels of (B) TGF-β1, Rac1, β-catenin, (D) E-cadherin and vimentin. β-actin was used as an internal control. The relative fold changes in mRNA or protein levels compared to the controls were calculated. Data represent the means ± SD from three independent experiments. *P<0.05 or **P<0.01 vs. control.

(Amersham Biosciences, Buckinghamshire, UK) and bands were visualized on X-ray film (Kodak, Rochester, NY, USA). Membranes were re-incubated with anti-β-actin antibody to verify equal protein-sample loading. The target protein amounts were normalized towards β-actin quantity using densitometry, then relative fold changes in protein levels were calculated as ratios between treated vs. control group values.

Cell migration and invasion assays. Invasion assays were performed using Transwell® plates (Corning, Inc., Corning, NY, USA) as previously described (12). Briefly, MGC803 cells were seeded onto Matrigel-coated filters (8-μm pore size), then were treated with TGF-β1 (5 ng/ml) or DADS (30 mg/l) alone, or incubated with TGF-β1 + SB431542 (10 μmol/l) or TGF-β1 + NSC23766 (50 μmol/l) for 24 h or left untreated. The cells that had invaded the lower surface of the filter were fixed and stained with hematoxylin. Invasiveness was determined by counting cells in four microscopic fields per well, and the extent of invasion was expressed as an average number of cells per microscopic field. Invasion rates were expressed as the ratio of the treated group value to the control group value. Transwell migration assays were conducted using the same procedure as for the invasion assay, except using the Matrigel-uncoated filters.

Gastric tumor growth in nude mice. Transfected and untransfected MGC803 cells were injected into the subcutis of the right axillary of male athymic BALB/c nude mice (4 weeks old). The mice were purchased from the Experimental Center of the Chinese Academy of Science in Shanghai. The mice were housed in an environment controlled for temperature (22±2˚C), light (12 h light/dark cycle) and humidity (60±10%). The animals were maintained under specific pathogen-free conditions in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The animals were randomly divided into six groups, and each group consisted of five mice. The mice were treated with normal saline, DADS (100 mg/kg) (11), SB431542 (10 mg/kg) (14) and NSC23766 (5 mg/kg) (15) via intraperitoneal injection every 2 days until the termination of the experiment. Tumor volume (cm^3) was examined every 6 days and calculated using a standard formula ($width^2$ x length x 0.5). Average tumor volumes are presented (n=5 for each group)

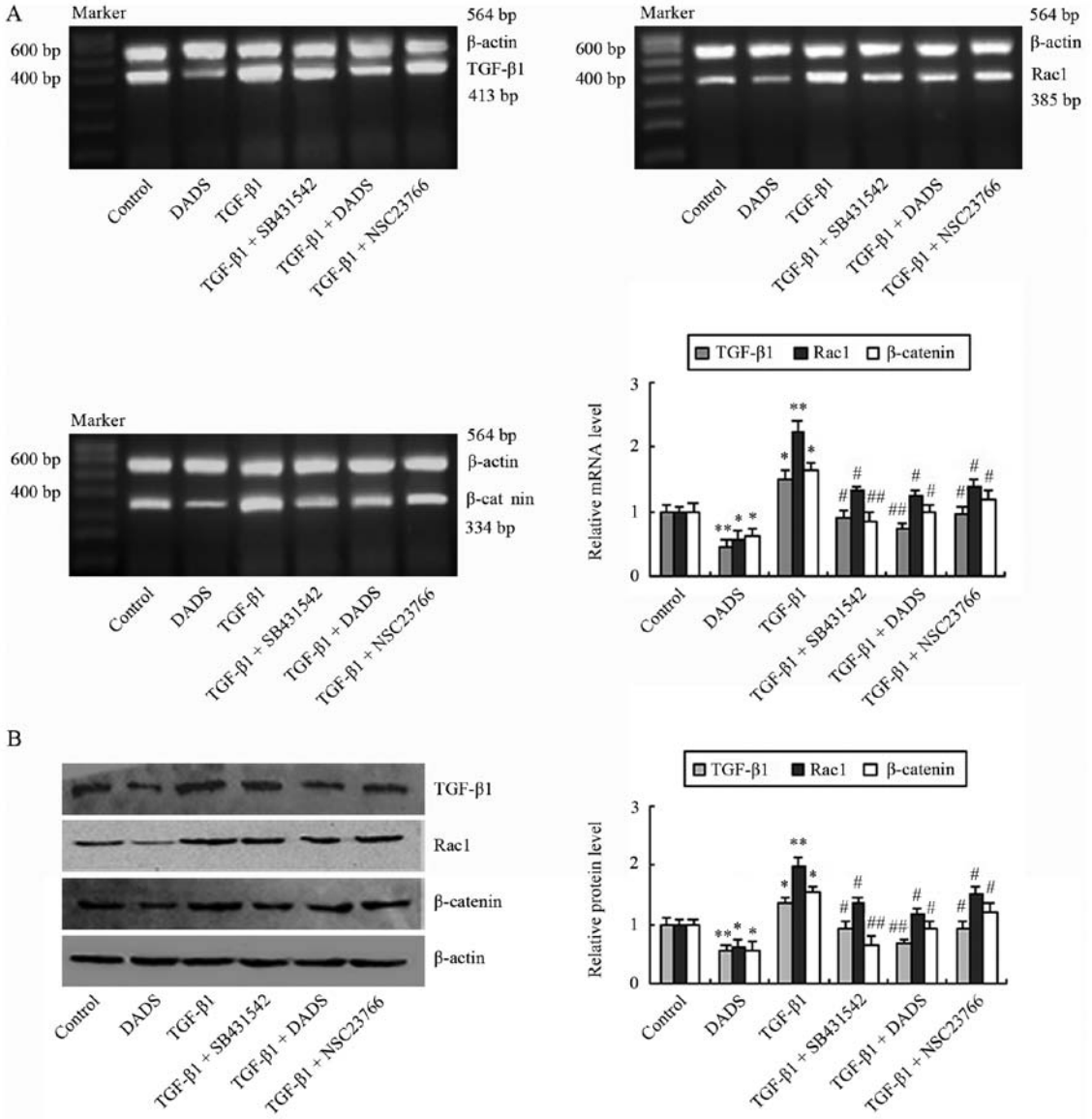

Figure 2. DADS inhibits the expression of Rac1 and β-catenin through downregulation of TGF-β1 in MGC803 cells. Cells are untreated or treated with DADS (30 mg/l), TGF-β1 (5 ng/ml), TGF-β1 (5 ng/ml) + SB431542 (10 μmol/l), TGF-β1 (5 ng/ml) + DADS (30 mg/l) and TGF-β1 (5 ng/ml) + NSC23766 (50 μmol/l) for 24 h. (A) RT-PCR was performed to detect the mRNA levels of TGF-β1, Rac1 and β-catenin. β-actin was used as an internal control for normalization. (B) Western blot analysis was performed to detect the protein levels of TGF-β1, Rac1 and β-catenin. β-actin was used as an internal control. The relative fold changes in mRNA or protein levels compared to the controls were calculated. Data represent the means ± SD from three independent experiments. *P<0.05 or **P<0.01 vs. control, $^{\#}$P<0.05 or $^{\#\#}$P<0.01 vs. the TGF-β1 group.

starting from the twelfth day and continuing until mice were sacrificed at 48 days by cervical dislocation under anesthesia. The xenografts were removed and the tumor size and weight were assessed at 48 days. Tumor tissues were then fixed in formalin and embedded in paraffin. Tissue sections (5 μm-thick) were prepared for subsequent immunohistochemistry analysis. All experiments were performed according to the guidelines for animal use of the Ethics Committee of the University of South China.

Immunohistochemistry. Briefly, after slides were dewaxed in xylene and hydrated in graded alcohol solutions, antigen retrieval was performed by heat treatment in 10 mM sodium citrate buffer (pH 8.0). Slides were incubated in 3% H_2O_2 solution to quench endogenous peroxidase activity and then incubated with normal goat serum for 20 min. Slides were incubated with primary antibodies (dilution 1:100) at 4˚C overnight. Positive signals were developed with peroxidase-conjugated secondary antibodies and 0.5% diaminobenzidine/H_2O_2 followed by counterstaining with Mayer's hematoxylin, dehydration, clearing and mounting. The slides that were treated with normal goat serum were evaluated as negative controls.

Statistical analysis. All results are presented as the mean ± SD for three independent experiments. Student's t-tests and one-way ANOVA were used to analyze the differences in expression among groups. P<0.05 were considered to indicate a statistically significant result. Statistical analyses were conducted using the SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA).

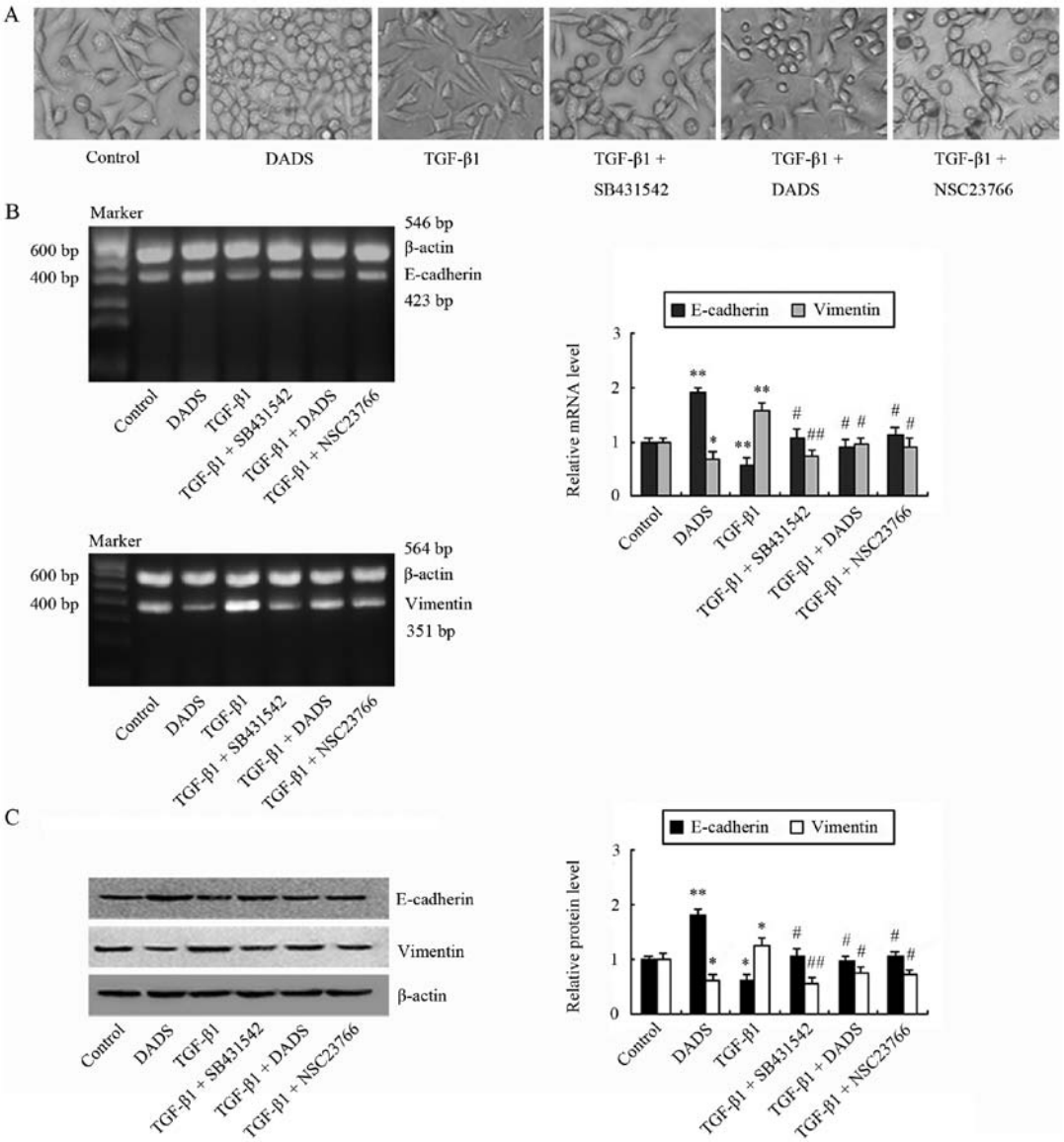

Figure 3. DADS suppresses TGF-β1-induced EMT and invasion by blocking TGF-β1 and Rac1. Cells are untreated or treated with DADS (30 mg/l), TGF-β1 (5 ng/ml), TGF-β1 (5 ng/ml) + SB431542 (10 μmol/l), TGF-β1 (5 ng/ml) + DADS (30 mg/l), and TGF-β1 (5 ng/ml) + NSC23766 (50 μmol/l) for 24 h. (A) Representative images were captured by phase-contrast microscopy to evaluate changes in cell morphology. (B) RT-PCR was performed to detect the mRNA levels of E-cadherin and vimentin. β-actin was used as an internal control for normalization. (C) Western blot analysis was performed to detect the protein levels of E-cadherin and vimentin. β-actin was used as an internal control. The relative fold changes in mRNA or protein levels compared to the controls were calculated. Data represent the means ± SD from three independent experiments. *P<0.05 or **P<0.01 vs. control, $^{\#}$P<0.05 or $^{\#\#}$P<0.01 vs. the TGF-β1 group.

Results

DADS downregulates TGF-β1, Rac1, β-catenin and vimentin and upregulates E-cadherin in MGC803 cells. We first treated cells with 30 mg/l DADS for different time-points and we examined the effects of DADS on the expression of TGF-β1, Rac1 and β-catenin. The mRNA and protein levels of TGF-β1 were reduced after cells were treated with DADS for 12, 24 and 48 h in a time-dependent manner (Fig. 1A and B). In contrast, Rac1 and β-catenin were decreased in the mRNA and protein levels after incubation for 24 h (Fig. 1A and B). These data indicated that DADS can reduce the expression of TGF-β1, Rac1 and β-catenin. In addition, we observed that a decrease in TGF-β1 occurred after 12 h of incubation, which was earlier than the decreases in Rac1 and β-catenin (24 h). We proposed that downregulation of TGF-β1 by DADS may result in the expression changes of its downstream effectors, Rac1 and β-catenin. The expression of E-cadherin in the mRNA and protein level was increased by DADS after 12 h of treatment. In contrast, the mRNA and protein levels of vimentin were reduced (Fig. 1C and D).

DADS antagonizes TGF-β1-induced upregulation of TGF-β1, Rac1 and β-catenin. Subsequently, we explored the effects of TGF-β1 on the expression of TGF-β1, Rac1 and β-catenin in MGC803 cells. The expression levels were determined after the cells were incubated with TGF-β1 (5 ng/ml) for 24 h. The mRNA and protein expression levels of TGF-β1, Rac1 and β-catenin were elevated in cells exposed to TGF-β1 (Fig. 2). The results indicated that TGF-β1 can induce

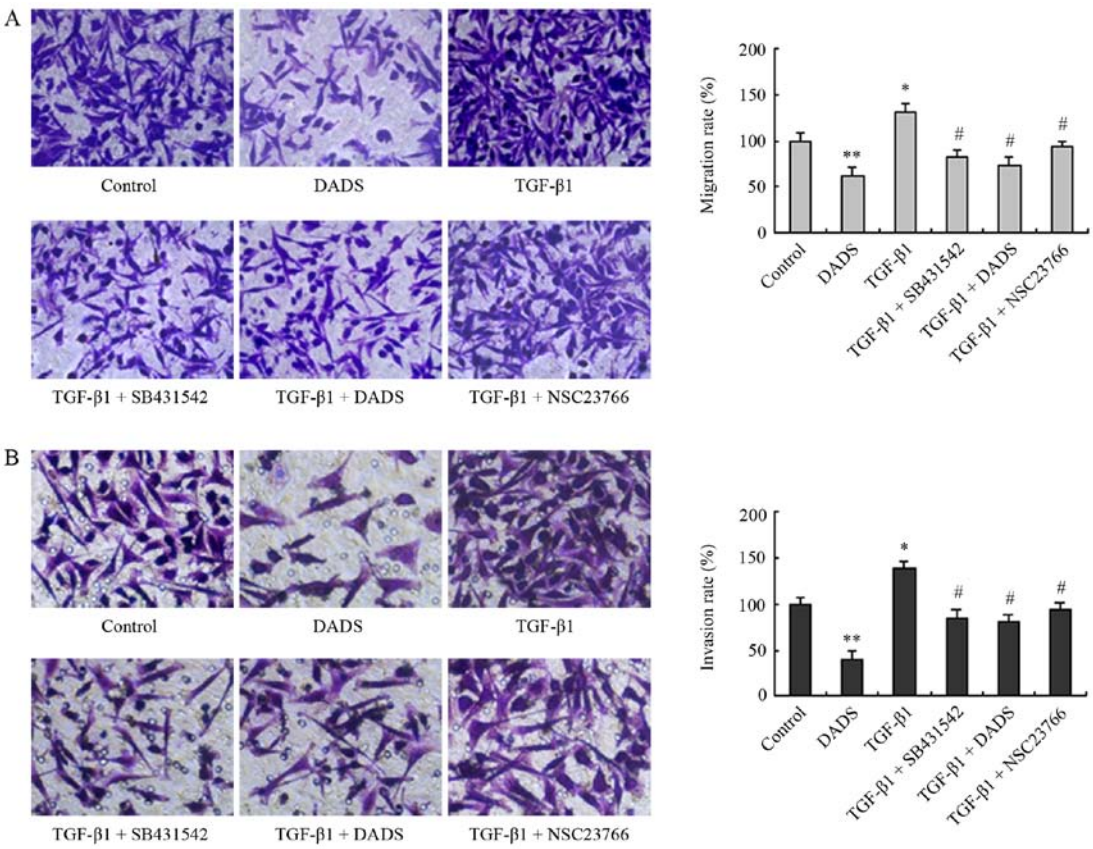

Figure 4. DADS suppresses TGF-β1-induced cell migration and invasion by blocking TGF-β1 and Rac1. (A and B) Cells were untreated or treated with DADS (30 mg/l), TGF-β1 (5 ng/ml), TGF-β1 (5 ng/ml) + SB431542 (10 μmol/l), TGF-β1 (5 ng/ml) + DADS (30 mg/l) and TGF-β1 (5 ng/ml) + NSC23766 (50 μmol/l) for 24 h. (A) Migration and (B) invasion rates were determined by the ratio of the mean number of cells between treated and untreated cells. Data represent the means ± SD from three independent experiments. *P<0.05 or **P<0.01 vs. control, $^{\#}$P<0.05 vs. the TGF-β1 group.

upregulation of TGF-β1, Rac1 and β-catenin. Compared with the TGF-β1-treated group, TGF-β1, Rac1 and β-catenin protein levels were decreased in the TGF-β1 + SB431542 group (Fig. 2B). Similarly, these protein levels were decreased after cells were treated with TGF-β1 in the presence of a Rac1 inhibitor, NSC23766. DADS (30 mg/l) treatment produced similar effects to those of SB431542 and NSC23766 (Fig. 2).

DADS suppresses TGF-β1-induced EMT and invasion by blocking TGF-β1 and Rac1. We observed that TGF-β1 treatment induced a morphological change (spindle-like morphology) and a decrease in cell-cell junctions, compared with the control group (Fig. 3A). In line with these morphological changes, an increase of vimentin and a decrease of E-cadherin in the mRNA and protein levels were demonstrated in the TGF-β1-treated group (Fig. 3B and C). Conversely, SB431542 and NSC23766 reversed these changes of morphology and EMT markers, which occurred in TGF-β1-treated cells (Fig. 3B and C). DADS exerted similar effects as SB431542 and NSC23766, decreasing vimentin and increasing E-cadherin, concomitantly with significant inhibition of morphological changes similar to mesenchymal cells (Fig. 3).

Subsequently, we further demonstrated that TGF-β1 treatment increased the rates of cell migration and invasion, while DADS neutralized these effects of TGF-β1, as did SB431542 and NSC23766 (Fig. 4). These data indicated that Rac1 mediated EMT induced by TGF-β1, whereas downregulation of TGF-β1/Rac1 signaling by DADS resulted in inhibition of EMT, migration and invasion.

DADS, TGF-β1 receptor inhibitor SB431542 and Rac1 inhibitor NSC23766 suppress TGF-β1-induced tumor growth in vivo. We have previously reported that DADS inhibits tumor growth by downregulating LIMK1, a downstream effector of Rac1 (11). We constructed a TGF-β1-overexpressing MGC803 cell line that exhibited increased TGF-β1 expression compared to the empty vector group and the control group (Fig. 5A). The transfected and untransfected cells were subcutaneously injected into nude mice. We examined the effect of TGF-β1 on tumor growth in nude mice, and determined whether the suppression of TGF-β1/Rac1 by DADS led to inhibition of gastric cancer MGC803 cell proliferation *in vivo*. The mice were subjected to different treatments and the tumor volume was examined every 6 days. Compared to the control group, the TGF-β1 group demonstrated an increase in tumor volume, whereas a decreased tumor volume was observed in the DADS group (Fig. 5B). The TGF-β1 + DADS, TGF-β1 + SB431542 and TGF-β1 + NSC23766 groups exhibited reduced tumor volumes, compared to the TGF-β1 group (Fig. 5B). After 48 days, the xenografts were removed from the mice. Similar changes were observed in tumor volume and weight (Fig. 5C and D). These data indicated that DADS antagonized TGF-β1-induced tumor growth via the downregulation of TGF-β1/Rac1 signaling.

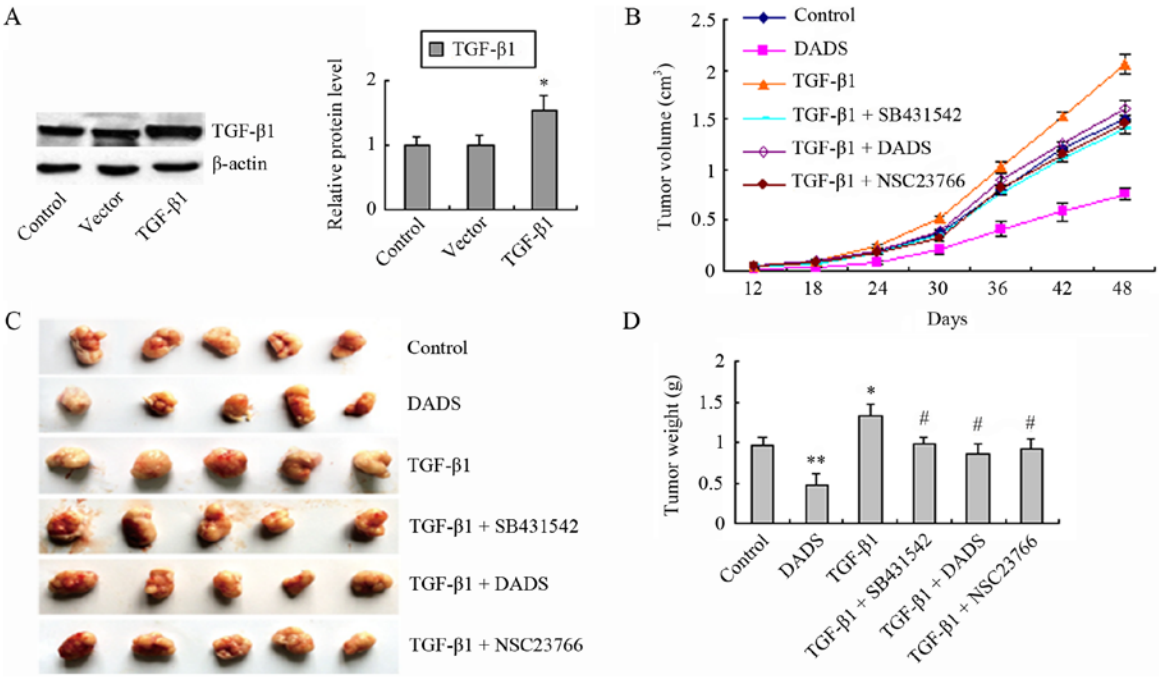

Figure 5. DADS, TGF-β1 receptor inhibitor SB431542 and Rac1 inhibitor NSC23766 suppress TGF-β1-induced tumor growth *in vivo*. (A) Cells were transfected with TGF-β1-expressing or empty vectors and western blot analysis was performed to determine the TGF-β1 protein expression level. β-actin was used as a loading control. The relative fold changes compared to the control group were calculated. Data represent the means ± SD from three independent experiments. *P<0.05 vs. the control group. (B) Untransfected, TGF-β1-expressing MGC803 cells were injected into the subcutis of nude mice. The mice of the untransfected group were treated with normal saline or DADS (100 mg/kg) and the mice of the transfected group were treated with normal saline, DADS (100 mg/kg), SB431542 (10 mg/kg) or NSC23766 (5 mg/kg) via intraperitoneal injection every 2 days. Tumor volume was assessed every 6 days. Average tumor volumes are represented (n=5 per group) starting from the twelfth day and continuing until sacrifice at 48 days. (C) The xenografts were collected at 48 days. Tumor sizes are shown for each group of MGC803 model mice. (D) The mean ± SD tumor weight for each group (n=5 per group) was calculated at the termination of the experiment. *P<0.05 or **P<0.01 vs. the control group, $^{\#}$P<0.05 vs. the TGF-β1 group.

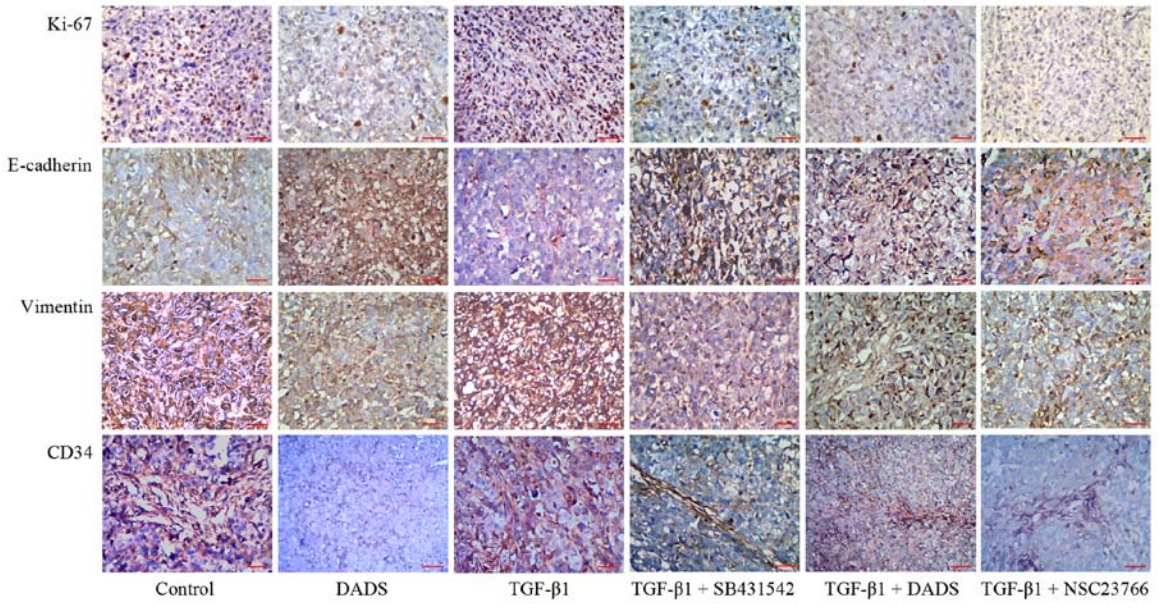

Figure 6. Effects of DADS on TGF-β1-induced E-cadherin, vimentin, Ki-67 and CD34 expression *in vivo*. Untransfected, TGF-β1-expressing MGC803 cells were injected into the subcutis of nude mice. The mice of the untransfected group were treated with normal saline or DADS (100 mg/kg) and the mice of the transfected group were treated with normal saline, DADS (100 mg/kg), SB431542 (10 mg/kg) or NSC23766 (5 mg/kg) via intraperitoneal injection every 2 days. The xenografts were collected at 48 days. Immunohistochemistry was performed to detect the expression of vimentin, E-cadherin, CD34 and Ki-67 in the tumor tissue specimens obtained from the xenografts. A representative tissue section is shown for each group (magnification, x400).

Effects of DADS on TGF-β1-induced E-cadherin, vimentin, Ki-67 and CD34 expression in vivo. We detected the protein expression levels of E-cadherin, vimentin, Ki-67 and CD34 in transplanted tumor tissues using immunohistochemistry. DADS reduced vimentin, Ki-67 and CD34 protein levels, and increased the expression of E-cadherin (Fig. 6). These results

were consistent with our previous data (11). The opposite effects were observed in the TGF-β1 group. SB431542 and NSC23766 attenuated the inhibitory effect of TGF-β1 on E-cadherin expression and weakened the enhanced effects of TGF-β1 on vimentin, Ki-67 and CD34 expression. Furthermore, DADS exerted the same effects on the expression of these proteins as these inhibitors (Fig. 6).

Discussion

TGF-β1 downregulates the expression of E-cadherin and upregulates the expression of vimentin, inducing EMT in gastric cancer cells (6). SB431542 (a TGF-β1 receptor inhibitor) blocks the TGF-β1 signaling pathway (16,17) and reverses TGF-β1-induced EMT in esophageal cancer cells, by downregulating the expression of N-cadherin and vimentin and upregulating the expression of E-cadherin (17). In the present study, we demonstrated that DADS decreased the expression of TGF-β1 and exerted same effects as SB431542, which abolished the enhanced effects of TGF-β1 on EMT and invasion in gastric cancer cells. Therefore, DADS inhibited EMT through the downregulation of TGF-β1 in gastric cancer cells.

We observed that Rac1 and β-catenin expression levels were increased in TGF-β1 treated gastric cancer cells which indicated that TGF-β1 may positively regulate the expression of Rac1 and β-catenin. DADS and SB431542 abrogated the TGF-β1 induced upregulation of the expression of Rac1 and β-catenin which indicated that downregulation of TGF-β1 by DADS resulted in reduced Rac1 and β-catenin expression. Rac1 (10) and β-catenin (7) pathways are involved in TGF-β1-induced EMT. DADS inactivates the β-catenin pathway and inhibits EMT in breast cancer cells (9). TGF-β1 induces EMT in prostate cancer cells via the activation of the Rac1/Pak1 pathway (10). DADS downregulates the Rac1/LIMK1 pathway, inhibiting EMT in gastric cancer cells (11). The decreased expression of Rac1 and β-catenin may contribute to the suppression of TGF-β1-induced EMT in gastric cancer cells.

We revealed that TGF-β1 promoted the expression of TGF-β1, while DADS and the TGF-β1 receptor inhibitor antagonized this effect. Furthermore, NSC23766, a Rac1 specific inhibitor, decreased the expression of TGF-β1 in TGF-β1-treated cells, indicating that Rac1 also positively regulated the expression of TGF-β1. NSC23766 inactivates Rac1 and results in abolishing colon cancer cell migration and invasion (18). Cigarette smoke extract (CSE)-induced EMT in pulmonary epithelial cells is associated with elevated Rac1 expression and increased TGF-β1 release and Rac1 inhibition by NSC23766 or knockdown decreases TGF-β1 release and abolishes CSE-induced EMT (19). TGF-β1 can induce the upregulated expression of Rac1 (20). Thus, a reciprocal positive interplay in expression regulation may exist between Rac1 and TGF-β1, and DADS may inhibit this positive feedback regulation mechanism.

There exists crosstalk between Rac1 and Wnt/β-catenin pathways. Rac1 acts as an upstream regulator of β-catenin. Overexpression of Rac1 augments Wnt3a-stimulated transcription of β-catenin target genes (21). Rac1-mediated JNK2 activation by Wnt3a promotes β-catenin phosphorylation and nuclear localization in ST2 cells (22). Rac1/PAK1 is required for the superactivation of β-catenin in colon cancer cells (23), and Rac1 promotes the formation of nuclear β-catenin-lymphoid enhancer factor 1 (LEF-1) complexes (24). Even without Wnt stimulation, Rac1 can still recruit β-catenin to its target genes and act as a co-activator in β-catenin/TCF (T cell factor)-mediated transcription in colon cancer cells (25). Furthermore, the interaction of active or inactive Rac1 with β-catenin is required for the nuclear translocation of β-catenin and Rac1 can promote β-catenin target gene transcription in breast cancer cells (26). TGF-β1 induces Rac1 activation in prostate cancer cells (10). Rac1 inhibitors attenuate Wnt/β-catenin pathway in breast cancer cells, reducing cell migration and invasion (27). We hypothesized that the increased Rac1 expression by TGF-β1 may facilitate the expression and activation of β-catenin in gastric cancer cells, while the reduced expression of Rac1 by DADS may reverse this effect of TGF-β1.

Rac1-mediated activation of β-catenin regulates the expression of Snail and MMP9, whereas knockdown of Rac1 decreases the expression and activation of β-catenin, resulting in impairing trophoblast invasion (28), which indicates that Rac1 may regulate the expression of β-catenin in addition to activating β-catenin. TGF-β1-induced EMT is mediated by ERK-dependent β-catenin upregulation and nuclear translocation in renal tubular epithelial cells (7). Rac1-mediated ERK activation is involved in TGF-β1-induced EMT in keratinocytes (29). We previously verified that DADS can downregulate Rac1/LIMK1 (11) and ERK (12) pathways in gastric cancer cells. We revealed that DADS, SB431542 and NSC23766 prohibited TGF-β1-induced upregulation of the expression of β-catenin. These data indicated that DADS reduced the expression of β-catenin through downregulation of the TGF-β1/Rac1 pathway in gastric cancer cells, which may, in part, account for the inhibitory effects of DADS on TGF-β1-induced EMT and invasion.

TGF-β1-mediated activation of Rac1/Pak1 pathway is associated with prostate tumor xenograft growth (10). Downregulation of Rac1/Pak1/LIMK1 (11) and Wmt/β-catenin (13) pathways is associated with the growth inhibition of gastric cancer cells *in vitro* and *in vivo*. In *in vivo* experiments, we further verified that DADS can reverse TGF-β1-induced EMT by blocking the TGF-β1/Rac1 pathway, which was supported by the upregulation of vimentin and the downregulation of E-cadehrin. Ki-67 is widely used as a marker to assess cell proliferation (30) and the overexpression of Ki-67 is related to poor prognosis of patients with gastric cancer (31). CD34 is a specific angiogenic marker and its expression is modulated by Pak1 (32). We have demonstrated that the decreased expression of Ki-67 and CD34 due to DADS-induced downregulation of LIMK1 is in accordance with the tumor growth inhibition (11). We illustrated that TGF-β1-induced tumor growth was attenuated by TGF-β1 receptor inhibitor SB431542 and Rac1 inhibitor NSC23766, concomitantly with the reduced expression of Ki-67 and CD34 in the transplanted gastric tumor.

In conclusion, DADS inhibited EMT, invasion and growth of gastric cancer cells by decreasing TGF-β1 expression, concomitantly with reduced Rac1 and β-catenin expression. These data indicated that the downregulation of TGF-β1/Rac1 pathway may, in part, account for the molecular mechanisms through which DADS exerts anti-EMT and antitumor growth effects in gastric cancer.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

BS, JS and YZ conceived and designed the study. BS and JS were involved in drafting and revising the manuscript. BS and YZ performed the RT-PCR. JS performed the immunohistochemistry. ED, FL and TT performed the cell culture and western blot analysis. HX and YHW performed the cell migration and invasion assays. XZ and HL were involved in the acquisition and analysis of the data. HJ, XHA and QS reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experiments were performed according to the guidelines for animal use of the Ethics Committee of the University of South China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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