

Inhibition of lysyl oxidase expression by dextran sulfate affects invasion and migration of gastric cancer cells

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Abstract. In the present study, the effect of dextran sulfate (DS) on the metastasis and invasion of human gastric cancer cells and its key underlying mechanism were investigated. The levels of hypoxia-inducible factor 1 α (HIF-1 α), transforming growth factor β (TGF- β) and lysyl oxidase (LOX) expression were evaluated in human gastric cancer and peritumoral tissues by immunohistochemical analysis. Cell proliferation and apoptosis were also examined using the Cell Counting Kit-8 assay and flow cytometry. The effect of DS on the invasion and migration of BGC-823 cells was assessed using a Transwell assay. BGC-823 cells were divided into the control (phosphate-buffered saline-treated) and experimental (DS-treated) groups, and cultured for different times under hypoxic conditions. Subsequently, LOX and TGF- β expression levels in the cells were measured by immunocytochemistry, immunofluorescence, reverse transcription-quantitative polymerase chain reaction and western blot analysis. HIF-1 α , TGF- β and LOX expression levels were significantly higher in human gastric cancer tissues as compared with that in adjacent tissues. DS influenced cell proliferation and apoptosis in a dose-dependent manner. Furthermore, DS reduced the number of invaded and migrated cells. Under hypoxic conditions, DS reduced HIF-1 α , TGF- β and LOX expression levels in BGC-823 cells. After 12 h, the effect of combination of DS and β -aminopropionitrile (BAPN) on LOX and TGF- β protein levels was more significant compared with that of DS

or BAPN alone. Therefore, DS may inhibit the invasion and migration of human gastric cancer cells under hypoxic conditions by influencing LOX.

Introduction

Metastasis is the main reason of poor prognosis in gastric cancer patients (1). The roles of various key factors in cancer metastasis continue to be evaluated, and targeted drug discovery and selection are important for preventing and treating metastatic cancer (2). The results of a recent study have suggested that the epithelial-mesenchymal transition (EMT) is a key step in tumor metastasis (3). EMT represents the loss of cell polarity and cell-cell adhesion by epithelial cells, which results in transformation to a mesenchymal phenotype, as well as migration and invasion, providing a mechanism for cancer development (4). Peritoneal metastasis of cancer cells occurs due to decreased adhesion between cells, resulting in primary cancer cell lesions breaking free from the serosa in the abdominal cavity.

The tissue oxygen balance is an important condition for the regulation of cell proliferation and survival (5), while hypoxia is one of the most important initiators of EMT during metastasis. Hypoxia-inducible factor 1 α (HIF-1 α) is a specific transcription factor that serves an active role in hypoxia by connecting upstream and downstream genes, activating a wide range of factors in mammals (6). Hypoxia increases the expression levels of transforming growth factor β (TGF- β), lysyl oxidase (LOX), matrix metalloproteinase (MMP), vascular endothelial growth factor (VEGF) and the mesenchymal cell marker vimentin, while it decreases the expression level of the endothelial marker E-cadherin. This leads to decreased tumor cell adhesion and increased mobility, facilitating metastasis and invasion of tumor cells (7).

TGF- β signaling serves an important role in cancer cell EMT, metastasis and invasion (8). High TGF- β expression promotes extracellular matrix (ECM) instability, consequently affecting the activity of the ECM protein, LOX (9). The TGF- β /Smad4 pathway is closely associated with cancer tissue differentiation. However, Smad4 and TGF- β expression levels

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exhibit opposite effects on the survival prognosis of gastric cancer patients, with TGF- β expression positively correlated and Smad4 expression negatively correlated with survival (10). Through the promotion of the transfer process, serum TGF- β expression is significantly higher in patients with peritoneal metastasis as compared with that in patients without peritoneal metastasis, although no independent factor has been identified to date (11). It has also been reported that TGF- β and LOX are closely linked with the process of metastasis (12).

LOX is a copper-dependent amine oxidase that is considered to participate in the ECM. It serves a pivotal role in morphological and structural integrity, while LOX overexpression leads to ECM remodeling and sclerosis, which provides the basis for tumor migration and invasion (13). Furthermore, LOX is considered a hypoxic response gene that is closely associated with the migration and invasion of tumor cells (14). LOX overexpression upregulates Snail expression and downregulates E-cadherin expression under hypoxic conditions, thereby promoting EMT (15). It has been suggested that HIF regulates LOX expression during the metastasis of breast, head and neck cancer, and inhibition of HIF decreases LOX expression, since these pathways are closely associated (16). Another study reported that TGF- β signaling may upregulate LOX expression and promote cancer metastasis under hypoxic conditions (17). In addition, LOX interference significantly reduces TGF- β expression by upregulating VEGF expression and P38 phosphorylation, inhibiting cancer cell migration and invasion (18). Thus, LOX has a potential clinical value in the diagnosis of gastric cancer lymph node and peritoneal metastases (19).

Overall, HIF-1 α , TGF- β and LOX are closely correlated and serve key roles in cancer metastasis. Based on these findings, the use of dextran sulfate (DS) as an intervention drug was evaluated in the present study. Our previous study showed that DS reduced the peritoneal metastasis of BGC-823 gastric cancer cells in nude mice and decreased the number of nodules and tumor cell volume with downregulated expression of HIF-1 α and ITG β 1 expression in gastric cancer cells (20). However, the mechanism underlying the effect of DS on the peritoneal metastasis of gastric cancer cells remains unclear. The present study aimed to evaluate the influence of DS on human gastric cancer cell migration and invasion under hypoxic conditions. It is speculated that DS may inhibit the mechanism of action of gastric cancer peritoneal metastasis.

Materials and methods

Gastric cancer tissues. A total of 40 specimens from gastric cancer patients who had not received radiotherapy or chemotherapy were obtained from the Department of Pathology at the Ningxia People's Hospital (Yinchuan, China) between March 2015 and March 2016. Informed consent was obtained from all individuals participating in the present study. Gastric cancer was diagnosed by two pathologists according to the ESMO-ESSO-ESTRO clinical practice guidelines (21). Gastric cancer and normal gastric peritumoral tissues were collected for immunohistochemical experiments.

Cell culture. The primary human gastric cancer BGC-823 cell line (Beijing Jin ZiJing, Beijing, China) was routinely

cultured in RPMI 1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/l streptomycin (HyClone; GE Healthcare Life Sciences). The cells were incubated in a humidified atmosphere at 37°C and 5% CO₂.

Drugs and cell groups. DS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in phosphate-buffered saline (PBS) for cell culture and then sterilized using a 22- μ m filter, to obtain a final concentration of 0.3%. β -aminopropionitrile (BAPN; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a final concentration of 300 μ M/ml was used. The appropriate concentrations of DS and BAPN for invasion and migration were obtained in the CCK-8 and flow cytometry (FCM) apoptosis assay under normoxic conditions. Then the cells were treated with PBS, DS, BAPN and DS combined with BAPN under hypoxia, respectively.

Cell Counting Kit-8 (CCK-8) assay. The effect of DS and BAPN on cell proliferation was determined using the CCK-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, cells in the logarithmic growth phase were seeded at a density of 1,000 cells/well into 96-well plates and cultured under normoxic conditions at 37°C for 24 h. Subsequently, the cells were treated with different concentrations of DS (0.1, 0.3, 0.6 and 1.0%) and BAPN (200, 300, 500, 700 and 1,000 μ M), and cultured for 24 h. Next, 10 μ l CCK-8 reagent was added to each well, and the cells were incubated at 37°C for an additional 3 h. The absorbance of each well, representing the cell viability, was measured at 450 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Flow cytometry (FCM) apoptosis assay. BGC-823 cells were seeded at a density of 5x10⁵ cells/well in a 6-well plate, treated with different DS concentrations (0.1, 0.3, 0.6 and 1.0%) and incubated for 24 h. The re-suspended cells were treated with 400 μ l Annexin V-fluorescein isothiocyanate (FITC) binding buffer (BestBio, Shanghai, China) and digested in 0.25% trypsin without ethylenediaminetetraacetic acid. The cells were centrifuged at 1,000 x g at room temperature for 5 min and washed three times with cold PBS. Annexin V-FITC (5 μ l) was applied for 20 min, followed by the addition of propidium iodide (10 μ l) and incubation for 5 min at 4°C. The stained cells were immediately analyzed by FCM (BD Biosciences, San Jose, CA, USA).

In vitro invasion and migration assays. BGC-823 cells were subjected to serum deprivation for 24 h and seeded at a density of 2.5x10⁴ cells/well on the top of Matrigel-coated filters. Cells were then transferred to chambers containing 600 μ l of 10% FBS as a chemo-attractant and incubated with DS (0.3%) and BAPN (300 μ M) under oxygen-deprived conditions for 24 h (22). The non-migrating or non-invading cells in the upper chamber were removed with a cotton swab. The invading cells were fixed with 4% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 15 min at room temperature, stained with 0.1% crystal violet and manually counted under a phase-contrast microscope at

Table I. Oligonucleotide primers for quantitative polymerase chain reaction.

Gene	cDNA primer sequence (5'-3')	Annealing temperature (°C)	Product size (bp)
TGF- β	Forward: GACACCAACTATTGCTTCAG Reverse: CAGGCTCCAAATGTAGGG	59	156
Smad4	Forward: CCCCATCTGAGTCTAATGCTAC Reverse: GCAGTCCTACTTCCAGTCCAG	60	217
LOX	Forward: TTGAGTCCTGGCTGTTATGATAACC Reverse: TGATGTCCTGTGTAGCGAATGTC	60	179
β -actin	Forward: TGACGTGGACATCCGCAAAG Reverse: CTGGAAGGTGGACAGCGAGG	60	284

LOX, lysyl oxidase; TGF- β , transforming growth factor β .

x400 magnification. The mean of each assay for six randomly selected fields was considered as the sample value. Experiments were repeated three times. The *in vitro* cellular migration assay was based on the described membrane invasion culture system, but with absence of Matrigel coating in the filters used.

Immunocytochemical analyses. Cells were seeded on the culture dish at a density of 30×10^4 /dish and treated with DS for 24 h under hypoxia as aforementioned. Then the cells were fixed in 4% paraformaldehyde (PFA) for 15 min and incubated in hydrogen peroxide for 15 min. The cells were then blocked with goat serum at 37°C for 30 min. Following incubation with anti-HIF-1 α (1:100; 20960-1-AP; ProteinTech Group, Inc., Wuhan, China), anti-TGF- β (1:150; ab27969; Abcam, Cambridge, MA, USA) and anti-LOX (1:150; ab174316, Abcam) and β -actin (1:1,000; E-AB-30422; Elabscience Biotechnology Co., Ltd., Wuhan, China).

Primary antibodies at 4°C overnight, the samples were incubated with goat anti-rabbit secondary antibodies (Histostain™- SP kits, SPN-9001; OriGene Technologies, Inc., Rockville, MD, USA) at room temperature for 1 h. Signals were detected using 3,3'-diaminobenzidine tetrahydrochloride (1:20; OriGene Technologies, Inc.), and the cells were counterstained with hematoxylin. The staining intensity score criteria were as follows: 0, no staining; 1, light yellow staining; 2, yellow staining; and 3, brown staining. The following scores were assigned for different percentages tumor-positive cells: 0, negative; 1, 1-25% positive cells; 2, 25-50%; and 3, >50%. The staining intensity, percentage of positive samples and tumor grades were scored between 0 and 9 (with 0 indicating a lack of brown immunoreactivity and 9 reflecting intense dark brown staining) (23), and divided into the following categories: 0-1, negative; 2, +; 3-4, ++; and ≥ 5 , +++, corresponding to low, moderate and high expression, respectively.

Immunofluorescence analysis. Cells were seeded on the culture dish at a density of 30×10^4 /dish and treated with DS for 24 h under hypoxia as aforementioned. Then, the cells were fixed in 4% paraformaldehyde at 37°C for 15 min and permeabilized with 0.5% Triton X-100 for 30 min. The cells were then blocked with goat serum at 37°C for 30 min. Following incubation with TGF- β (1:150) and LOX (1:150) with primary antibodies overnight at 4°C, followed by incubation with

FITC-conjugated and tetramethylrhodamine-conjugated secondary antibodies, all samples were counterstained with 4',6-diamidino-2-phenylindole (BIOSS, Beijing, China) and examined under an fluorescence microscope (IX73PIF, Olympus Corporation, Tokyo, Japan). Images were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-PCR) analysis. Total RNA of treated cells was extracted from cells after treatment. Next, RNA was reverse transcribed into cDNA using a total mRNA extraction kit (Total RNA kit I, R6834-01; OMEGA, Guangzhou, China) and an RevertAid RT kit (K1691; Sangon Biotech Co., Ltd., Shanghai, China), according to the manufacturer's protocols. qPCR was subsequently performed using PCR mixture/kit (2XTaq MasterMix, CW0682; CWBio, Beijing, China). with the cDNA primer sequences provided in Table I. qPCR was performed under the following conditions: 94°C for 2 min, 94°C 30 sec, 58°C for 30 sec, 72°C for 30 sec under 30 cycles, 7°C for 2 min and hold at 4°C. For qPCR, the mRNA expression levels of TGF- β , Smad4 and LOX were analyzed using target/internal relative grayscale values (ImageJ software 1.48u; National Institutes of Health, Bethesda, MD, USA) based on the expression level of 18S rRNA QuantumRNA™ Technology in Multiplex Quantitative RT-PCR using 18S rRNA as an Internal Control. (QuantumRNA™ Classic 18S Internal Standard, AM1716; Thermo Fisher Scientific, Inc.).

Western blot analysis. Total proteins were isolated by Total Extraction Kit (KGP2100; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) following the manufacturer's protocols. Protein concentration was determined by the BCA reagent kit (KGPBCA; Nanjing KeyGen Biotech Co., Ltd.). Equal amounts of protein (60 μ g) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes, which were blocked in 5% non-fat milk, and then incubated with the primary antibodies against anti-HIF-1 α (1:1,000; ProteinTech Group, Inc.), anti-TGF- β (1:500; Abcam, USA), anti-Smad4 (1:1,000; ProteinTech Group, Inc.), anti-LOX (1:2,000; Abcam, USA), anti-E-cadherin (1:200; Cell Signaling Technology, Danvers, MA, USA) and anti- β -actin (1:1,000; Elabscience Biotechnology Co., Ltd.). The samples were incubated with the

antibodies overnight at 4°C. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:6,000; OriGene, Beijing, China) at room temperature for 1 h. An enhanced chemiluminescence reagent (ECL kit, KGP1121; Nanjing KeyGen Biotech Co., Ltd.) was applied as a chromogenic substrate for 1 min, and then visualized with an Amersham Imager 600 instrument (GE Healthcare Life Sciences). Grayscale analysis was performed with ImageJ software.

Statistical analysis. All experiments were assayed in triplicate (n=3). Data are expressed as the mean ± standard error of the mean. All statistical analyses were performed using SPSS version 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). Two-sample comparison was performed using t-test. Multiple samples were compared using one-way analysis of variance. The correlation between LOX and TGF-β was analyzed using four samples and three replications by Pearson's correlation analysis. Differences with a P<0.05 were considered to exhibit a statistically significant difference.

Results

HIF-1α, TGF-β and LOX expression levels in poorly differentiated gastric carcinoma and peritumoral tissues. Immunohistochemical staining revealed that HIF-1α was mainly located in the nucleus of cells in gastric carcinoma tissues (Fig. 1A), whereas TGF-β and LOX were mainly located in the cytoplasm (Fig. 1B and C) as indicated by positively stained brown particles or clusters. The levels of HIF-1α, TGF-β and LOX expression were markedly higher in gastric cancer as compared with those in peritumoral tissues (Table II).

Effects of DS on BGC-823 cell proliferation and apoptosis. To determine the toxic effects of DS and BAPN on BGC-823 cells, the same number of BGC-823 cells was incubated with different concentrations of DS and BAPN, and cell survival was assessed using the CCK-8 assay. The relative cell survival rates were 89.9, 77.2, 70.0 and 57.8% at DS concentrations of 0.1, 0.3, 0.6 and 1.0%, respectively, while the survival rates were 94.6, 72.7, 81.8, 76.6 and 73.7% at BAPN concentrations of 200, 300, 500, 700 and 1,000 μM, respectively (Fig. 2A and B). In addition, the combined use of DS (0.3%) and different concentrations of BAPN (200, 300 and 500 μM) resulted in relative survival rates of 86.6, 66.9 and 64.4%, respectively (Fig. 2C). Importantly, 0.3% DS combined with 300 μM BAPN exerted an effective inhibition effect on BGC-823 cell than 300 μM BAPN. FCM was also used to detect the effect of different DS concentrations on apoptosis under the same conditions. When the DS concentrations were 0.1, 0.3, 0.6 and 1.0%, the relative apoptosis rates were 10.5, 14.1, 13.7 and 16.1%, respectively (Fig. 2D). The FCM and CCK-8 assay results demonstrated the dose dependence of the DS effect. Furthermore, these data indicate that BGC-823 apoptosis and survival are influenced by DS and BAPN in a dose-dependent manner. For subsequent analysis, 0.3% DS was selected as the optimal concentration.

Effects of DS on BGC-823 cell migration and invasion. First, cells were treated with 0.3% DS and/or 300 μM BAPN for 24 h under hypoxia, and incubation with these continued throughout

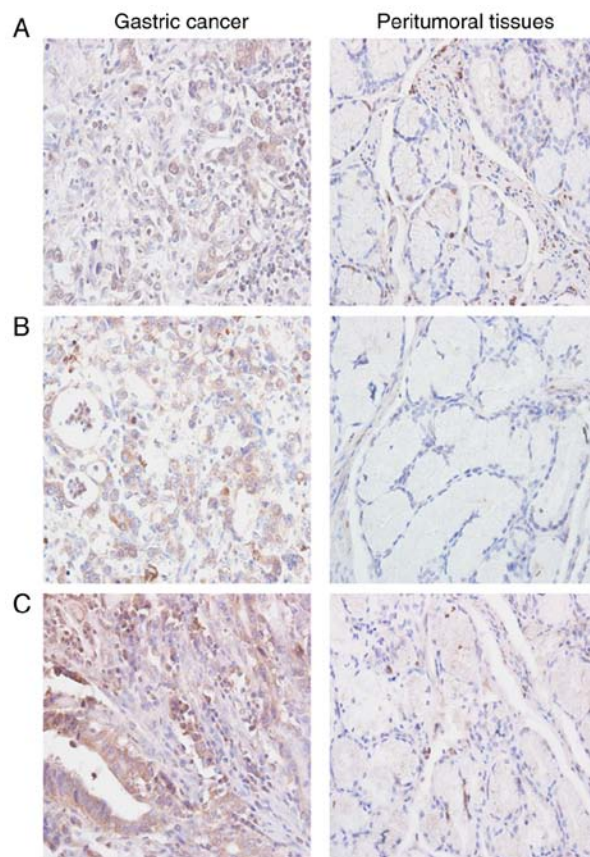


Figure 1. Immunohistochemical analysis of (A) HIF-1α, (B) TGF-β and (C) LOX expression in poorly differentiated gastric cancer and peritumoral tissues. Original magnification, x400. HIF-1α, hypoxia-inducible factor 1α; TGF-β, transforming growth factor β; LOX, lysyl oxidase.

the experiment. Subsequently, invading and migrating cells were fixed, stained with crystal violet and counted. Both DS and BAPN were observed to significantly reduce the invasion and migration of BGC-823 cells in comparison with the control group. Compared with DS or BAPN alone, the combination of DS and BAPN resulted in significantly reduced cell invasion and migration (Fig. 3).

Immunocytochemical staining. LOX was observed to be mainly expressed in the cytoplasm of BGC-823 cells and in a few nuclei, with brown staining indicating positive LOX expression (Fig. 4A). Positive TGF-β expression was detected in the nucleus and cytoplasm by brown staining with high expression in the nucleus. (Fig. 4B). The LOX and TGF-β expression levels were significantly decreased at 2 h (P<0.05), 8 h (P<0.05), 12 h (P<0.01) and 24 h (P<0.01) of incubation with DS, as compared with the corresponding control group (Fig. 4C and D). Thus, these results indicate that DS inhibited LOX and TGF-β expression levels in BGC-823 cells.

Immunocytofluorescence analysis of LOX and TGF-β expression levels. The current study attempted to further elucidate the association between LOX and TGF-β expression levels in gastric cancer cells under hypoxic conditions by immunocytofluorescence analysis (Fig. 5A and B). LOX expression in the DS group was significantly reduced at 2 h (P<0.05), 8 h (P<0.01), 12 h (P<0.01) and 24 h (P<0.05) when compared

Table II. HIF-1 α , TGF- β and LOX expression levels in poorly differentiated gastric cancer and peritumoral tissues.

Tissues	Number	HIF-1 α ⁺ (%)	TGF- β ⁺ (%)	LOX ⁺ (%)
Gastric cancer	40	26 (65) ^a	31 (77.5) ^b	29 (72.5) ^b
Peritumoral	40	16 (40)	13 (32.5)	14 (35)

^aP<0.05 and ^bP<0.01 vs. HIF-1 α , TGF- β and LOX expression in peritumoral tissues. +, indicates tumor-positive percentages of >50%. HIF-1 α , hypoxia-inducible factor 1 α ; TGF- β , transforming growth factor β ; LOX, lysyl oxidase.

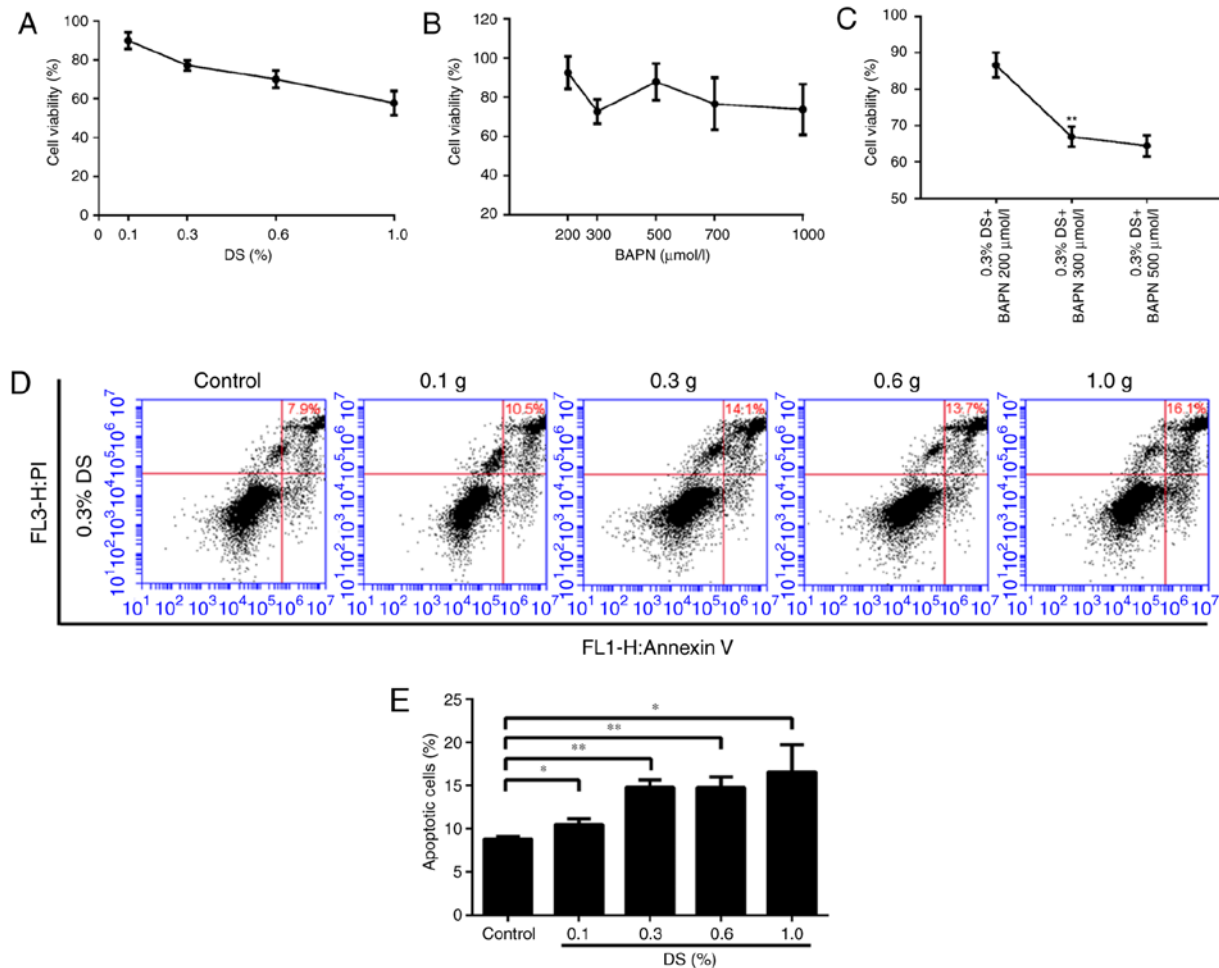


Figure 2. Effects of different DS concentrations on the proliferation and apoptosis of gastric cancer cells. Under normoxic conditions, Cell Counting Kit-8 was used to detect the BGC-823 cell proliferation ability. Relative survival rate of cells exposed to different concentrations of (A) DS (0.1, 0.3, 0.6 and 1.0%) and (B) BAPN (200, 300, 500, 700 and 1,000 μ M). (C) Effect of the combination of 0.3% DS and BAPN (200, 300, and 500 μ M). **P<0.01 vs. 300 μ M BAPN. (D) Flow cytometry analysis and (E) percentage of apoptotic cells. The induction of apoptosis in human gastric cancer cells was determined by flow cytometry following treatment with DS (0.1, 0.3, 0.6 and 1.0%) for 24 h. *P<0.05 and **P<0.01, vs. control group. DS, dextran sulfate; BAPN, β -aminopropionitrile.

with that in the control group (Fig. 5C). Similarly, the level of TGF- β expression was significantly lower at 2 h (P<0.05), 8 h (P<0.05), 12 h (P<0.01), and 24 h (P<0.01) in the DS group in comparison with that in the control group (Fig. 5D). This suggested that, under hypoxia, DS may inhibit the expression TGF- β ; however LOX expression levels may be inhibited in a time dependent manner.

RT-PCR analysis of LOX, TGF- β and Smad4 mRNA expression levels in BGC-823 cells. As LOX, TGF- β and Smad4 serve a significant role in the process of invasion and migra-

tion and is a prognostic indicator of gastric cancer (10,19), the present study investigated their expression profiles. The RT-PCR results revealed that the mRNA expression levels of LOX (8 and 12 h, P<0.01) and TGF- β (2, 8 and 12 h, P<0.01) were significantly decreased, while the expression of Smad4 (2 and 8 h, P<0.01) was significantly increased in BGC-823 cells under hypoxic conditions at certain time points in the DS group, when compared with the levels in the control group (Fig. 6). According these results, DS may regulate the expression of LOX, TGF- β and Smad4 thereby inhibiting the invasion and migration of gastric cancer cells.

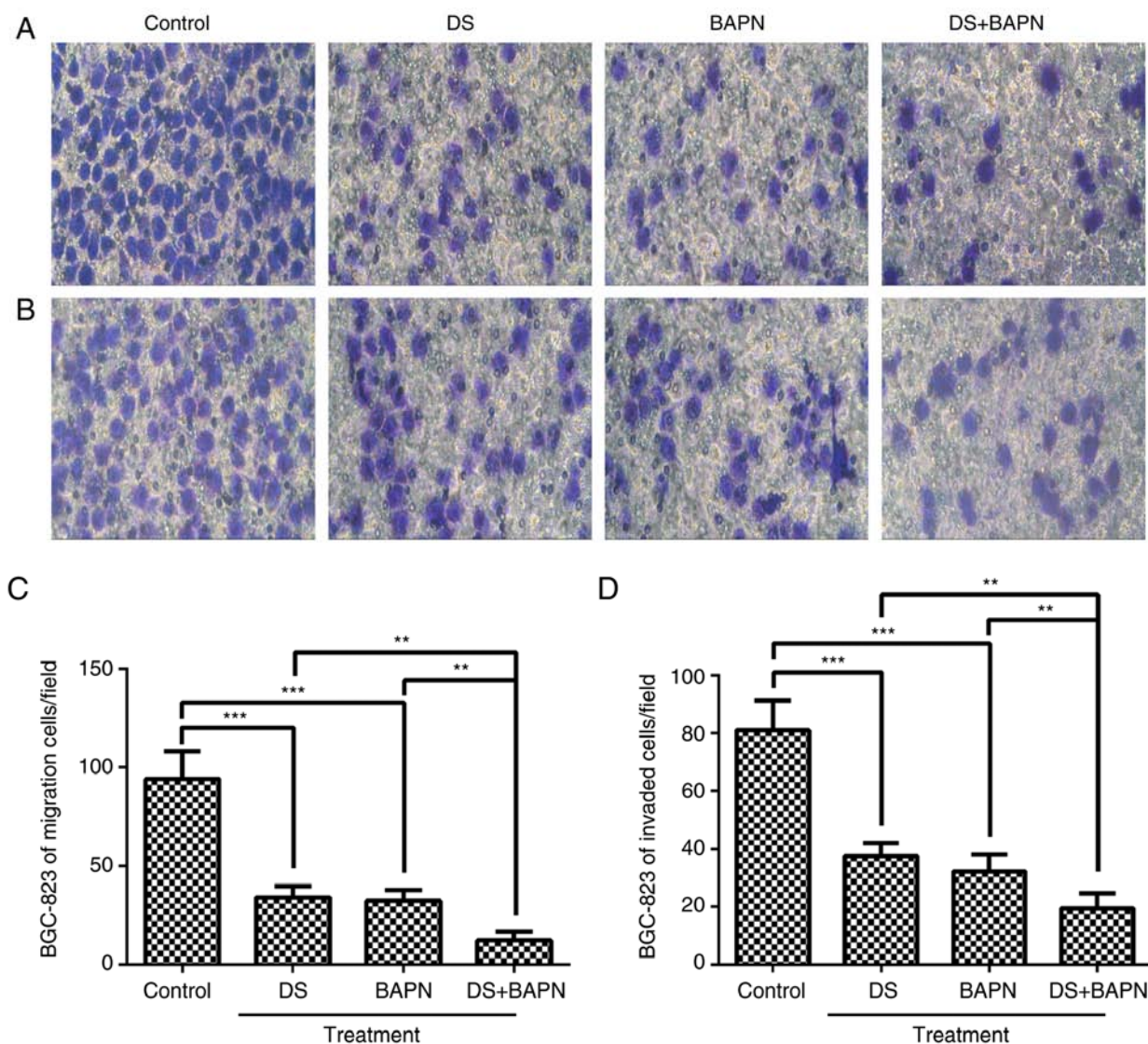


Figure 3. DS and BAPN inhibited hypoxia-induced (A) migration and (B) invasion of BGC-823 cells. (C) Migrating and (D) invading cell percentages are shown. Cells were treated with 0.3% DS and/or 300 μ M BAPN for 24 h prior to hypoxia, and then migration and invasion assays were conducted. Invading and migrating cells were fixed, stained with crystal violet and counted. Data are expressed as the mean \pm standard error of the mean. ** P <0.01 and *** P <0.001. Original magnification, x400. DS, dextran sulfate; BAPN, β -aminopropionitrile.

Effect of DS on metastasis-associated protein expression levels in BGC-823 cells. Western blot analysis was conducted to determine the levels of various metastasis-associated proteins (Fig. 7A). Following treatment with 0.3% DS, LOX, HIF-1 α and TGF- β protein expression levels were significantly decreased at 8, 12 and 24 h compared with in the control group (Fig. 7B-D). In contrast, the expression levels of Smad4 were significantly increased at 2 and 8 h in response to DS treatment compared with in the control, but no alterations were observed at 12 and 24 h following treatment of DS (Fig. 7E). In addition, the expression levels of E-cadherin at 2, 8, 12 and 24 h were significantly upregulated under hypoxia and DS compared with control; (Fig. 7F).

Furthermore, the inhibitory effects of 0.3% DS and 300 μ M BAPN on LOX, HIF-1 α and TGF- β were enhanced when the cells were treated by DS in combination with BAPN, compared with DS or BAPN, respectively. This suggested that there may be a synergistic inhibition of BGC-823 cells between DS and BAPN (Fig. 8). Additionally, the correlation between LOX

and TGF- β expression levels were investigated using Person's correlation analysis. This indicated that the expression of LOX was positively associated with TGF- β (Table III).

Discussion

Metastasis and recurrence are major causes of mortality in gastric cancer patients, and tumor molecular markers are important for determining the prognosis and predicting the recurrence of tumors (24). Despite improvements in surgical approaches and chemotherapy regimens for the treatment of gastric cancer, patients with metastasis and recurrence still have a poor prognosis. Therefore, limiting tumor metastasis is the key to effectively increasing patient survival rates.

Several signaling factors are expressed at higher levels in cancerous tissue as compared with their expression in tissues adjacent to the tumor. Therefore, these signaling factors have an important clinical value in the study of cancer metastasis. It has been suggested that high HIF-1 α expression under hypoxic

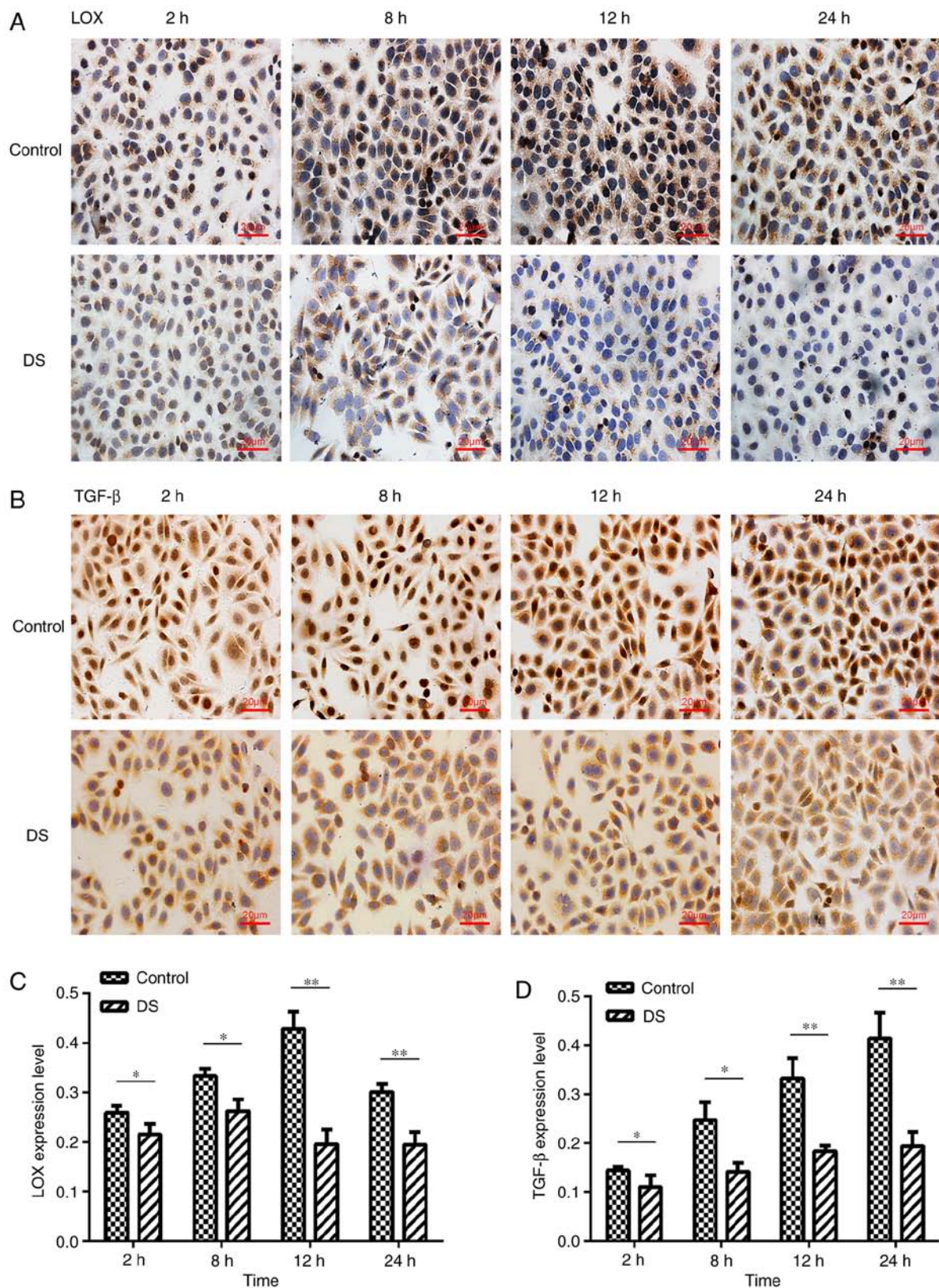


Figure 4. Immunohistochemical analysis of (A) LOX and (B) TGF- β expression in BGC-823 cells. The expression levels of (C) LOX and (D) TGF- β in the control and DS groups under hypoxic conditions are shown at different time points (2, 8, 12 and 24 h). Data are presented as the mean \pm standard error. * P <0.05 and ** P <0.01. Original magnification, x400. DS, dextran sulfate; LOX, lysyl oxidase; TGF- β , transforming growth factor β .

conditions can promote tumor cell angiogenesis, EMT, invasion and migration (25). A previous study demonstrated that DS can prevent B16 melanoma cells from implanting on greater omentum milky spots and the peritoneum, thus

servicing an important role in the inhibition of abdominal cavity metastasis (26). When DS was introduced into the murine visceral endoderm through endocytosis, the endoderm cells maintained normal functions and their unique membrane

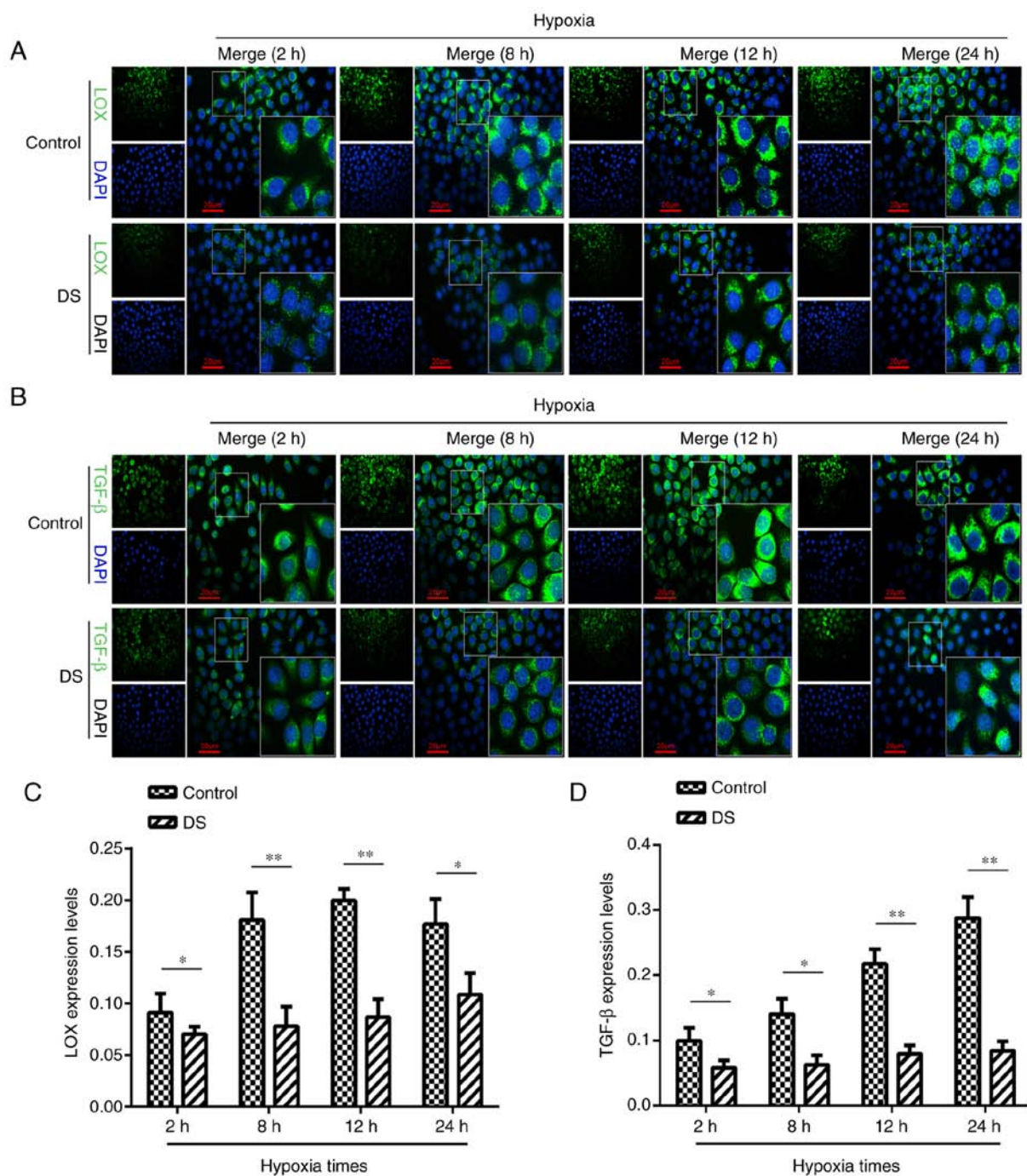


Figure 5. (A) LOX and (B) TGF- β expression levels in the DS and control groups, as detected by immunofluorescence. (C) LOX and (D) TGF- β quantified expression levels are also shown. Under hypoxic conditions, LOX and TGF- β were expressed in the cytoplasm and nucleus. In the control group, expression of the two proteins increased with time, while the DS group exhibited significantly reduced expression levels of the two proteins. Data are presented as the mean \pm standard error. * $P < 0.05$ and ** $P < 0.01$. Original magnification, $\times 400$. DS, dextran sulfate; LOX, lysyl oxidase; TGF- β , transforming growth factor β .

dynamics (27). The present study demonstrated that DS inhibited hypoxia-induced BGC-823 cell migration and invasion under hypoxic conditions, while significantly reducing the high level of HIF-1 α expression.

Under anoxic conditions, LOX is closely connected with key signaling pathways that are important in the cancer metastasis process (28). In eukaryotic cells, HIF-1 α mainly adjusts the oxygen balance and, in turn, is regulated by oxygen. A hypoxic environment exists in the majority of tumors due to tumor growth-induced blood vessel anfractuosity, which alters the tumor microenvironment. Despite the rich oxygenation

state of growth in surrounding tissues, a growth state with relatively low oxygenation exists in the central organization of tumor tissues (29). HIF-1 α has been demonstrated to increase LOX, Twist and Snail expression levels, and to reduce E-cadherin expression under hypoxic conditions, thereby accelerating cancer cell migration and invasion *in vitro* (30). In addition to HIF-1 α , TGF- β is another key factor that promotes LOX expression (12) and is the key regulatory factor in cell proliferation, apoptosis, differentiation and migration processes, as well as in ECM synthesis and precipitation (31). In the present study, it was observed that TGF- β was mainly

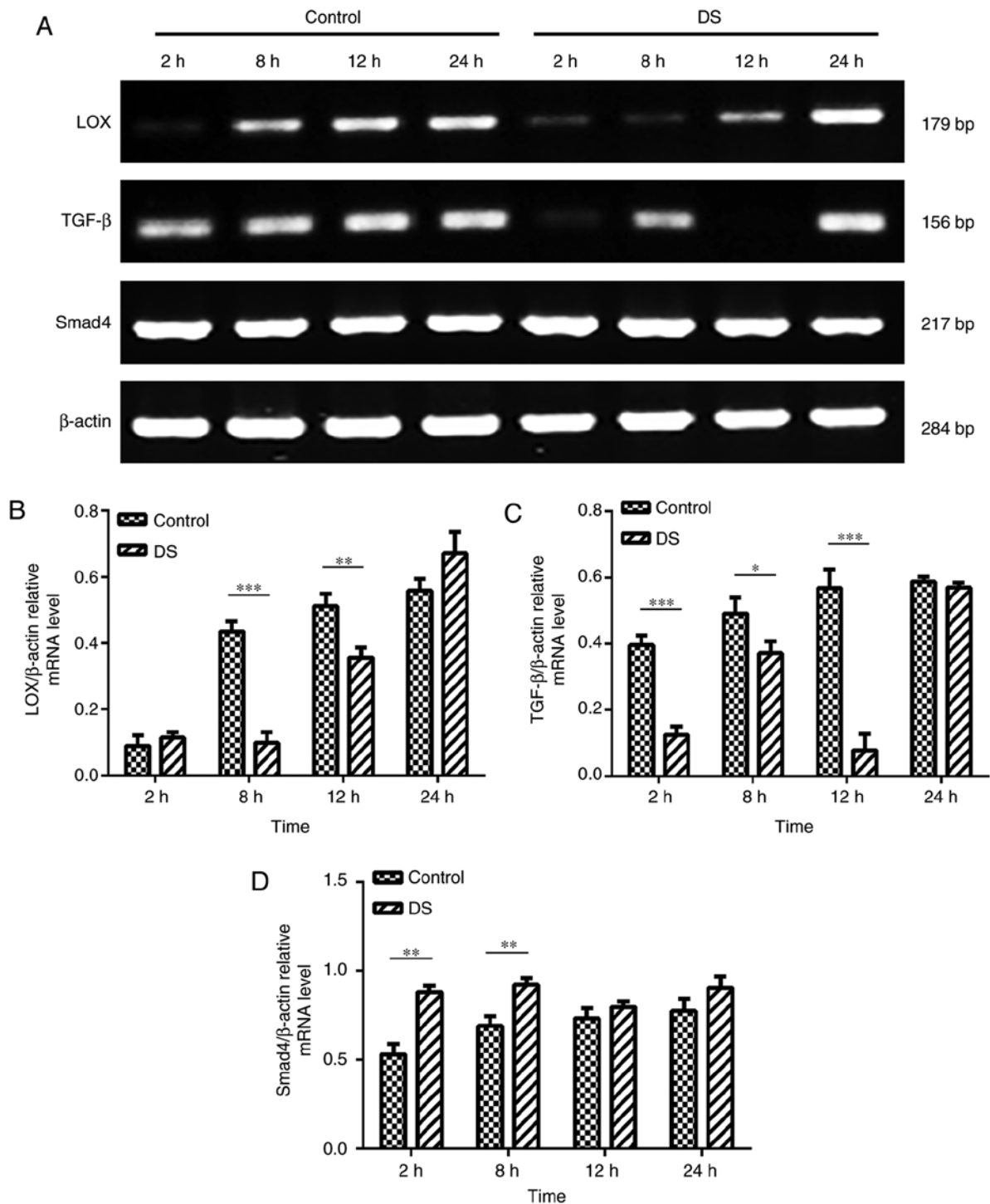


Figure 6. LOX, TGF- β and Smad4 mRNA expression levels in BGC-823 cells. (A) Reverse transcription-quantitative polymerase chain reaction was performed, and the quantified mRNA expression levels of (B) LOX, (C) TGF- β and (D) Smad4 are shown. Under hypoxic conditions, LOX and TGF- β mRNA levels increased with time, whereas decreased expression was observed in the DS group. The Smad4 mRNA expression level was increased in the DS group compared with that in the control group. Data are presented as the mean \pm standard error. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. DS, dextran sulfate; LOX, lysyl oxidase; TGF- β , transforming growth factor β .

expressed in the nucleus and cytoplasm under hypoxia and that TGF- β was able to translocate from the cytoplasm to nucleus. DS reduced TGF- β expression at different time points, with significantly reduced nuclear expression, suggesting that DS can inhibit the nuclear translocation of TGF- β . Under hypoxic conditions, TGF- β protein expression increased gradually, whereas this expression was decreased after 24 h. It has previously been reported that the development of cancer TGF- β

signaling has a dual role in tumor suppression and promotion (32); therefore, the present study speculated that TGF- β may affect BGC-823 cell autocrine secretion through other unknown pathways.

Studies on cancer metastasis have reported that TGF- β is closely linked to LOX expression. TGF- β signaling increases LOX expression to promote breast cancer cell metastasis. However, the capacity of cancer cell metastasis was found to

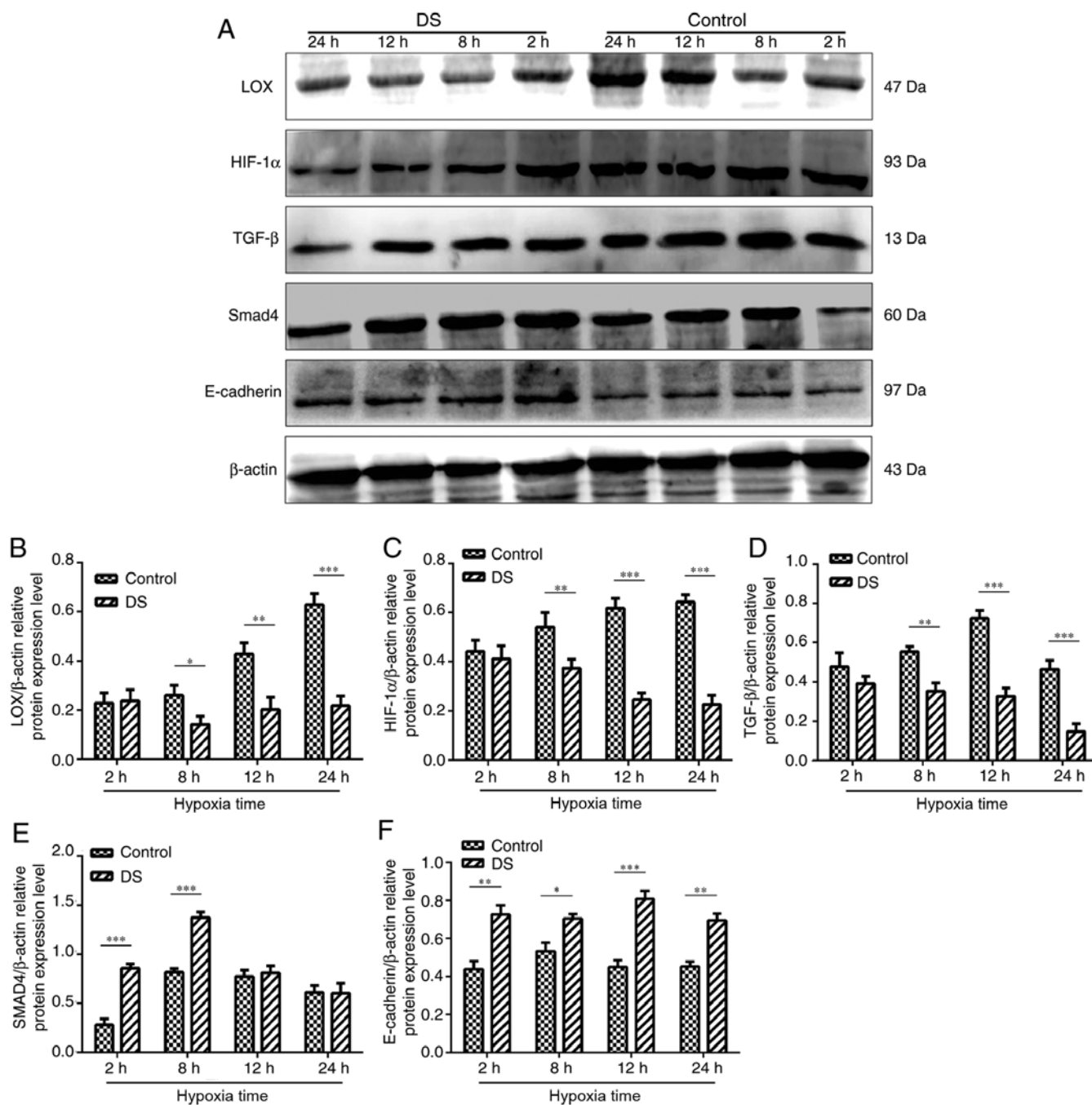


Figure 7. (A) Western blot analysis of HIF-1 α , TGF- β , LOX, Smad4 and E-cadherin protein expression levels in BGC-823 experimental and control cell groups. (B) LOX, (C) HIF-1 α and (D) TGF- β protein expression levels were significantly reduced in the DS group. By contrast, DS significantly increased (E) Smad4 and (F) E-cadherin protein expression compared with the control group. The western blot analysis results were analyzed using ImageJ software and are shown as bar graphs. Similar results were obtained in at least three independent experiments. Data are presented as the mean \pm standard error. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. DS, dextran sulfate; LOX, lysyl oxidase; HIF-1 α , hypoxia-inducible factor 1 α ; TGF- β , transforming growth factor β .

be reduced by inhibition of LOX activity (12). Another study reported that the application of a LOX inhibitor reduced the number of cells with a polygon or long spindle shape under hypoxic conditions (9). Although the experiments of the current study demonstrated that 0.3% DS reduced LOX expression, the BGC-823 cell morphology was not significantly altered (data not shown). One possible cause for this discrepancy may be due to the poor differentiation of BGC-823 cells (20).

Smad4 is a key factor in the TGF- β signaling pathway (33). It was initially identified as a tumor suppressor gene and as the central regulation factor of TGF- β signal transduction,

however, with the continuous progression of the tumor, the expression of Smad4 decreased (10). Hu *et al* (34) reported that a significantly higher TGF- β expression in gastric cancer tissues compared with that in para-carcinoma tissues, and continuous alterations in the tumor microenvironment accelerated the invasion and migration of gastric cancer cells in clinical pathological specimens. In addition, Leng *et al* (10) reported that Smad4 expression was higher in gastric cancer tissues, poorly differentiated carcinomas and patients without lymphatic metastasis in comparison with that in para-carcinoma tissues, highly differentiated gastric cancer and lymphatic metastases.

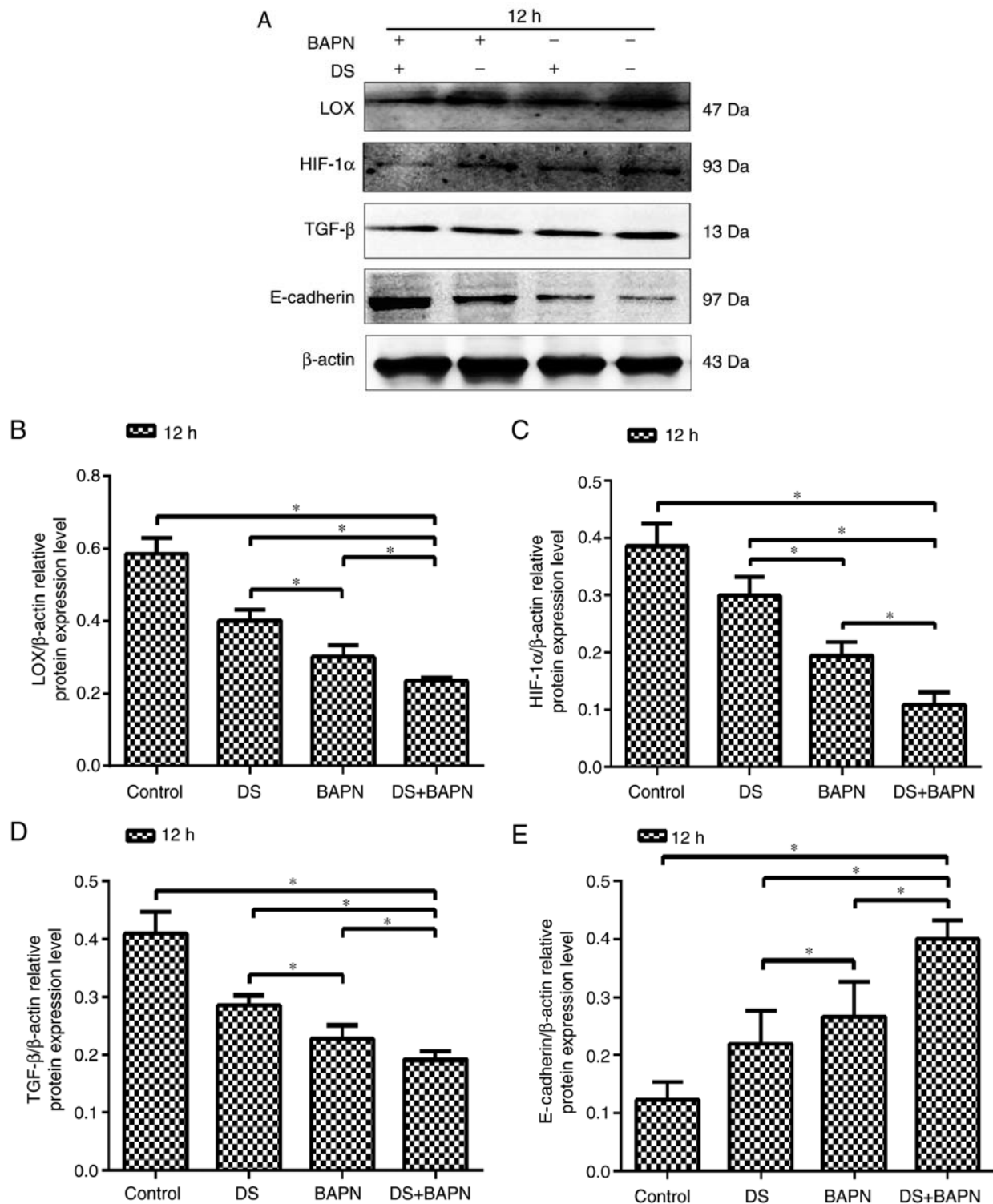


Figure 8. Combination of DS and BAPN inhibited LOX, HIF-1 α and TGF- β expression levels, and promoted E-cadherin expression. (A) Western blot analysis, and quantified results of the expression levels of (B) LOX, (C) HIF-1 α , (D) TGF- β and (E) E-cadherin at 12 h. β -actin was used as a loading control. The western blotting results were analyzed using ImageJ software and are shown as bar graphs. Data are presented as the mean \pm standard error. * P <0.05. DS, dextran sulfate; BAPN, β -aminopropionitrile; LOX, lysyl oxidase; HIF-1 α , hypoxia-inducible factor 1 α ; TGF- β , transforming growth factor β .

The present study found that DS significantly reduced TGF- β and increased Smad4 expression under hypoxic conditions. Therefore, it is speculated that DS can affect gastric cancer cell metastasis through the TGF- β /Smad signaling pathway.

In cancer research, inhibition of LOX expression can effectively reduce EMT and the migration of cancer cells. Under hypoxic conditions, the autocrine activity of LOX

increased Snail expression and enhanced invasive ability; indeed, cancer cell migration, invasion and EMT capacity were reduced following application of the LOX activity inhibitor BAPN (35). It has been demonstrated that BAPN inactivates LOX and decreases its upstream factors HIF-1 α and TGF- β , while it increases the expression of E-cadherin, which affects tumor cell metastasis (22). Furthermore, BAPN

Table III. Correlation between LOX and TGF- β protein levels *in vitro*.

Protein	Statistical value	LOX	TGF- β
LOX	Pearson's correlation	1	0.898 ^a
	P-value (two-sided)	-	<0.001
	n	4	4
TGF- β	Pearson's correlation	0.898 ^a	1
	P-value (two-sided)	<0.001	-
	m	4	4

^aP<0.01. LOX, lysyl oxidase; TGF- β , transforming growth factor β .

was reported to exert the most effective inhibitory effects on invasion and metastasis at a concentration of 500 μ M against low oxygen-induced ovarian cancer cell invasion and metastasis (36). In a breast cancer study, higher LOX expression occurred with a change in the tumor microenvironment, which promoted the early dissolution of bone lesions and cancer cell metastases (37). Furthermore, in a hepatocellular carcinoma study, TGF- β caused increased cancer cell expression of LOX and VEGF protein in a dose-dependent manner, and LOX gene silencing was able to decrease the phosphorylation of TGF- β expression by p38, indicating that LOX may influence the expression of TGF- β via the mitogen-activated protein kinase pathway (18). The involvement of LOX in the process of cancer metastasis has gradually emerged from a number of studies (9,38). In the current study, experiments focused on investigating the influence of DS on certain key factors during hypoxia-induced cancer cell migration. Under hypoxic conditions, LOX expression increased over time in the control group, but was significantly decreased by DS in a time-dependent manner, concomitant with an increase in E-cadherin expression. Preliminary experiments also revealed that DS may reduce cancer cell TGF- β and LOX expression and increase E-cadherin expression.

Notably, combined application of the LOX inhibitor BAPN (300 μ M) and 0.3% DS decreased HIF-1 α , TGF- β and LOX expression levels at 8 and 12 h, while it significantly increased Smad4 and E-cadherin expression levels. These results indicate that combined application of BAPN and DS is better than the single application of BAPN or DS. The current study also speculated that DS may inhibit the expression of LOX, thereby affecting TGF- β signaling in the invasion and migration of human gastric cancer cells. The DS-mediated inhibition of key signaling pathways should be further explored in the future to improve the prevention and treatment options for cancer metastasis. Although an inhibitory effect of DS on gastric cancer cell migration and invasion was demonstrated in the present study, further research is needed to determine whether this effect is generalizable to other cancer types.

In conclusion, the data presented in the current study revealed a positive correlation between LOX and TGF- β protein expression, and that DS may inhibit the invasion and migration of gastric cancer cells by inhibiting LOX expression under hypoxic conditions. Furthermore, the combined application of DS and BAPN had a more prominent effect. Elucidating

the mechanism through which DS inhibits intra-abdominal gastric cancer cell migration and its clinical application value require further analysis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YX conceived the design of the present study. XW wrote the manuscript with support from all the other authors. XW and XJ conducted cell culture, immunohistochemistry and immunofluorescence experiments. YH guided and performed CCK-8 and cell flow cytometry experiments. YM, XJ, and HW conducted western blotting and reverse transcription-quantitative polymerase chain reaction. JW performed immunofluorescence experiments and data analysis. All authors read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Ningxia Medical University (Yinchuan, China). Informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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