

# S100A9 promotes the proliferation and migration of cervical cancer cells by inducing epithelial-mesenchymal transition and activating the Wnt/ $\beta$ -catenin pathway

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Received October 1, 2018; Accepted April 17, 2019

DOI: 10.3892/ijo.2019.4793

**Abstract.** S100 calcium-binding protein A9 (S100A9), a member of the S100 protein family, is often upregulated in various cancers, including cervical cancer. Elevated S100A9 expression is thought to serve an important role in tumorigenesis; however, the exact role of S100A9 in the modulation of cervical cancer and the underlying molecular mechanism remain unknown. In the present study, we aimed to investigate the effects of S100A9 on the proliferation and migration of cervical cancer cells, as well as the molecular mechanisms underlying these effects. Our results demonstrated that endogenous expression of S100A9 in SiHa and CaSki cell lines was significantly higher than in the HeLa cell line. As expected, overexpression of S100A9 enhanced the proliferation and migration of cervical cancer cells. In addition, S100A9 overexpression induced epithelial-mesenchymal transition (EMT) as determined by reduced expression levels of the epithelial marker E-cadherin, whereas the expression levels of the mesenchymal marker vimentin were upregulated. Furthermore, it was reported that the effects of S100A9 in the modulation of cervical cancer cells were mediated through the Wnt/ $\beta$ -catenin signaling pathway as  $\beta$ -catenin knockdown significantly suppressed the ability of S100A9 to enhance the proliferation and migration of cervical cancer cells. Collectively, these findings suggest that S100A9 promoted

the proliferation and migration of cervical cancer cell lines. Furthermore, the underlying molecular mechanisms may be partially attributed to the induction of EMT and activation of the Wnt/ $\beta$ -catenin signaling pathway.

## Introduction

Cervical cancer ranks as the third most commonly diagnosed cancer and is the second leading cause of cancer-associated mortality in females worldwide (1). Despite improvements in screening and diagnostic techniques, regional or local relapse, and lymph node metastasis remain the most prevalent causes of cervical cancer-related mortality (2). Similarly, the motility and invasiveness of cancer cells serve a critical role in the mortality of patients with cervical cancer. Therefore, advances in understanding the mechanism of metastasis in cervical cancer may provide insight for developing effective approaches to prevent and treat cervical cancer.

S100A9, known as Calgranulin B or MRP-14, is a member of the low-molecular calcium binding S100 protein family. In our previous study, we demonstrated that exogenous S100A9 promoted the proliferation and invasion of HepG2 cells, which was partly mediated by activation of the MAPK signaling pathway (3) and that the underlying mechanism may be dependent on receptor of advanced glycation end products (RAGE)/MAPK signaling cascades (4). Interestingly, increasing evidence indicates that S100A9 is overexpressed in cervical cancer (5,6) and could be a candidate marker for the early diagnosis of cervical cancer and a potential therapeutic target for therapy (5). Few studies have investigated the precise role of S100A9 in the tumorigenesis and development of cervical cancer, and the underlying potential molecular mechanism.

Epithelial-mesenchymal transition (EMT) is a vital process associated with the pathogenesis of numerous cancers, including cervical cancer. This transition is characterized by a process in which epithelial cells lose their polarity enabling them to assume a mesenchymal cell phenotype, which imparts cancer

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**Key words:** S100A9, cervical cancer, proliferation, migration, EMT, Wnt/ $\beta$ -catenin signaling pathway

cells several properties, such as tumor invasion, carcinoma metastasis and chemotherapy resistance (7). A previous study demonstrated that cellular RAGE binding to S100A8/A9 can cause a notable increase and accumulation of endogenous Snail in the nucleus of breast cancer cells; thus, EMT and cancer metastasis are enhanced via the nuclear factor (NF)- $\kappa$ B signaling module (8). Additionally, the effects of transforming growth factor  $\beta$ 1 on EMT were antagonized by S100A9 via the formation of heterodimers between them in pancreatic cancer; however, the effects of S100A9 on EMT in cervical cancer require further investigation.

Recent studies reported that cytoplasmic/nuclear expression of  $\beta$ -catenin was associated with the malignant phenotype and the pathogenesis of cervical cancer (9,10). In addition, it was indicated that the Wnt/ $\beta$ -catenin pathway and EMT may act synergistically in the progression of cervical cancer (11). Tumor cells exhibiting  $\beta$ -catenin accumulation in the nucleus appear to promote EMT, which involves the loss of epithelial markers and the expression of mesenchymal markers (12). Members of the S100 protein family, including S100A6, S100B, and S100P were reported to negatively regulate  $\beta$ -catenin degradation. Our consensus finding is that recombinant S100A8 and S100A9 proteins could promote the accumulation of  $\beta$ -catenin, and upregulate mRNA expression of the target genes of the Wnt/ $\beta$ -catenin signaling pathway in colorectal carcinoma (13). However, whether S100A9 regulates the Wnt/ $\beta$ -catenin pathway, thereby exerting its effects on the progression of cervical cancer remains unknown.

In the present study, the functional role of S100A9 in the Wnt/ $\beta$ -catenin signaling pathway and EMT was investigated to elucidate how S100A9 modulates the progression of cervical cancer. We reported that S100A9 promoted the proliferation, migration, and EMT of cervical cancer cells, and the activation of the Wnt/ $\beta$ -catenin signaling pathway may be involved in the modulation of these processes. These findings provide novel insight to the importance of S100A9 in the progression of cervical cancer and a basis for the development of targeted therapeutic strategies for treating cervical cancer.

## Materials and methods

**Cell culture.** The human embryonic kidney cell line 293 and the human cervical cancer cell lines SiHa, CaSki, and HeLa were obtained from the American Type Culture Collection. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS; 10%; both obtained from Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (both obtained from HyClone; GE Healthcare) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. 293 cells were used for adenovirus amplification.

**Reagents and antibodies.** Adenovirus expressing S100A9 and green fluorescent protein (AdS100A9), adenovirus expressing small interfering (si)RNA targeting  $\beta$ -catenin and red fluorescent protein (Adsi $\beta$ -catenin), control adenovirus expressing green fluorescent protein (AdGFP) and red fluorescent protein (AdRFP), and pGST-moluc and pGST-moluc-hS100A9 plasmids were kindly donated

by Professor T.C. He (The University of Chicago Medical Center, Chicago, IL, USA). Competent bacteria *E. coli* (BL21) were saved in our laboratory. Adenoviruses expressing siRNA targeting S100A9 and red fluorescent protein (AdsiS100A9), and control adenoviruses expressing red fluorescent protein (AdsiControl) were constructed in house. The kit used for semi-quantitative PCR was purchased from Takara Bio, Inc. Antibodies, including mouse anti- $\beta$ -actin, anti-vimentin and anti- $\beta$ -catenin were purchased from Santa Cruz Biotechnology, Inc. (cat. nos. sc-47778, sc-66001 and sc-59737). Rabbit anti-S100A9 antibody was purchased from Abcam (cat. no. ab92507). Rabbit anti-E-cadherin antibody was purchased from ImmunoWay (cat. no. YM3353, Plano). Rabbit anti-histone H3 antibody was purchased from Abmart (cat. no. P30266). Secondary antibody reagents, such as goat anti-mouse IgG serum and goat anti-rabbit IgG serum were obtained from Beijing Zhongshan Golden Bridge Biotechnology (cat. no. 2305 and no. 2301). Western blot reagents and radioimmunoprecipitation assay (RIPA) buffer were purchased from Beyotime Institute of Biotechnology. Phosphatase and protease inhibitors were purchased from Roche Diagnostics GmbH. Polyvinylidene difluoride (PVDF) membranes and an enhanced chemiluminescence (ECL) kit were purchased from EMD Millipore.

**Adenovirus infection.** HeLa cells were infected with AdS100A9 and AdGFP, whereas SiHa cells were infected with AdsiS100A9 and AdsiControl. After 8-12 h of incubation, the medium was replaced with complete medium containing FBS followed by continued cell culture for subsequent experiments. The cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

**Recombinant protein preparation.** The pGST-moluc and pGST-moluc-hS100A9 plasmids used in the present study has been described previously (4). In brief, pGST-moluc and pGST-moluc-hS100A9 was transfected into *E. coli* (BL21) by calcium chloride-mediated transformation. Isopropylthio- $\beta$ -D-galactoside was used to induce the expression of GST and GST-hS100A9 proteins. The bacteria were then collected and sonicated on ice at 4°C. The supernatants were incubated with glutathione-sepharose 4B beads, GST and GST-hS100A9 proteins on the beads were eluted by elution buffer with reduced glutathione on ice. Finally the GST and GST-hS100A9 proteins were filtered and stored at -80°C. Cells were treated with 20  $\mu$ g/ml of recombinant proteins for 48 h at 37°C.

**RNA extraction and semi-quantitative polymerase chain reaction (PCR) analysis.** Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and cDNA samples were synthesized using random primers and a Reverse Transcriptase PCR kit (Takara Bio, Inc.) according to the manufacturer's protocols. cDNA was used as a template for semi-quantitative PCR. The thermocycling conditions were as follows: 94°C for 5 min, 94°C for 30 sec, 68°C for 30 sec and 72°C for 12 cycles with a decrease in 1°C/cycle; then, 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec for 18-27 cycles depending on the abundance of the target genes. The PCR products were identified by

Table I. Primers employed in the present study.

Gene	Primer sequences	Product size (bp)
S100A9	Forward: 5'-ACCCAGACACCCTGAACC-3' Reverse: 5'-AGCATGATGAACTCCTCGA-3'	163
c-Myc	Forward: 5'-TACCTCTCAACGACAGCAG-3' Reverse: 5'-TCTTGACATTCTCTCGGTG-3'	478
Snail	Forward: 5'-ACCCACATCCTTCTCACTG-3' Reverse: 5'-TACAAAAACCCACGCAGACA-3'	217
Twist	Forward: 5'-TCTTACGAGGAGCTGCAGAC-3' Reverse: 5'-TATCCAGCTCCAGAGTCTCT-3'	406
GAPDH	Forward: 5'-CAGCGACACCCACTCCTC-3' Reverse: 5'-TGAGGTCCACCACCTGT-3'	120

electrophoresis using 1.5% agarose gels. GAPDH was used as an internal reference control. The results were recorded using Gel Doc 1000 imaging system and Quantity One version 4.5.0 software (Bio-Rad Laboratories, Inc.). The primers used in this study are shown in Table I.

**Western blot analysis.** Total cellular protein of cervical cancer cells were prepared using RIPA buffer containing phosphatase and protease inhibitors. Nuclear and cytoplasmic proteins were extracted using Nuclear-Cytosol Extraction Kit (cat. no. KGP1100, Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's instructions. Protein extracts were separated by 6-15% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% bovine serum albumin at 37°C for 2 h, washed with Tris-buffered saline with Tween-20 (TBST) and incubated with primary antibodies against S100A9, vimentin, E-cadherin,  $\beta$ -catenin, histone H3, and  $\beta$ -actin separately (1:1,000 dilution) at 4°C overnight. After washing with TBST, the membranes were incubated with the a secondary antibody conjugated with horseradish peroxidase (1:5,000 dilution) for 1 h at 37°C. Finally, the blots were washed with TBST, and then visualized using the Bio-Rad Gel Doc 1000 imaging system and analyzed by Quantity One Version 4.5.0.

**Cell proliferation assay.** Cell proliferation was analyzed via an MTT assay (Promega Corporation). A total of  $3.5 \times 10^3$  HeLa cells or  $4.5 \times 10^3$  SiHa cells were seeded in 96-well flat-bottomed microplates. After infection with or without adenoviruses in DMEM containing 1% FBS for 24, 48 and 72 h, 10  $\mu$ l of MTT reagent was added to each well and incubated at 37°C for 4 h. Dimethyl sulfoxide (100  $\mu$ l) was added to dissolve the formazan crystals for 10 min at room temperature. Finally, the absorbance was measured at 490 nm using an automatic enzyme immunoassay analyzer (Bio-Rad Laboratories, Inc.). The experiments were performed five times in three independent experimental trials.

**Wound healing assay.** Cervical cancer cells were seeded in 6-well plates and incubated at 37°C until the cells attained 90% confluence. The cells were then infected with adenoviruses. Cell monolayers were carefully wounded by

scratching with a sterile 10- $\mu$ l pipette tip. The cells were then washed twice with cold PBS and incubated in serum-free DMEM at 37°C. Wound areas were observed and imaged at x100 magnification at 0, 24 and 36 h with an inverted fluorescent microscope (Nikon eclipse 80i; Nikon Corporation). Overall, the experiments were repeated three times.

**Transwell migration assay.** A chamber of non-type I-collagen-coated 24-well Transwell cell culture inserts (EMD Millipore) was used for the Transwell migration assay. Briefly, after infection with or without adenoviruses and recombination proteins for various durations, the cells were trypsinized, washed, and suspended in 400  $\mu$ l of serum-free DMEM and finally seeded in the upper chamber (HeLa,  $4 \times 10^4$  cells per well; SiHa,  $6 \times 10^4$  cells per well). The lower chamber was coated with 700  $\mu$ l of DMEM containing 20% FBS as a chemoattractant. After 24 h, cells were fixed with methanol for 20 min and stained with 0.05% crystal violet for 30 min at room temperature. Cells on the upper surface of the insert membrane were removed with cotton swabs. Finally, the cells were counted using an inverted microscope (magnification, x100) in five randomly selected fields for each well. The experiment was repeated three times.

**Immunofluorescence.** The cells were seeded on sterile glass coverslips in 24-well culture plates. After treatment with or without AdS100A9, cells were washed with PBS, fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.3% Triton X-100 at 37°C for 30 min. After blocking with goat serum at 37°C for 30 min, the cells were incubated with antibodies against E-cadherin, N-cadherin (1:100 dilution in PBS) at 4°C overnight. Subsequently, slides were washed with PBS and incubated with fluorescein isothiocyanate-conjugated secondary antibodies (Beyotime Institute of Biotechnology) for 1 h at room temperature in the dark. Then, the cells were washed with PBS, counter stained with DAPI (Beyotime Institute of Biotechnology) for 5 min at room temperature, washed with PBS again and then the coverslips were mounted using antifade mounting medium. The fluorescence of the various groups was visualized imaged with a Nikon eclipse 80i inverted fluorescent microscope (Nikon Corporation).

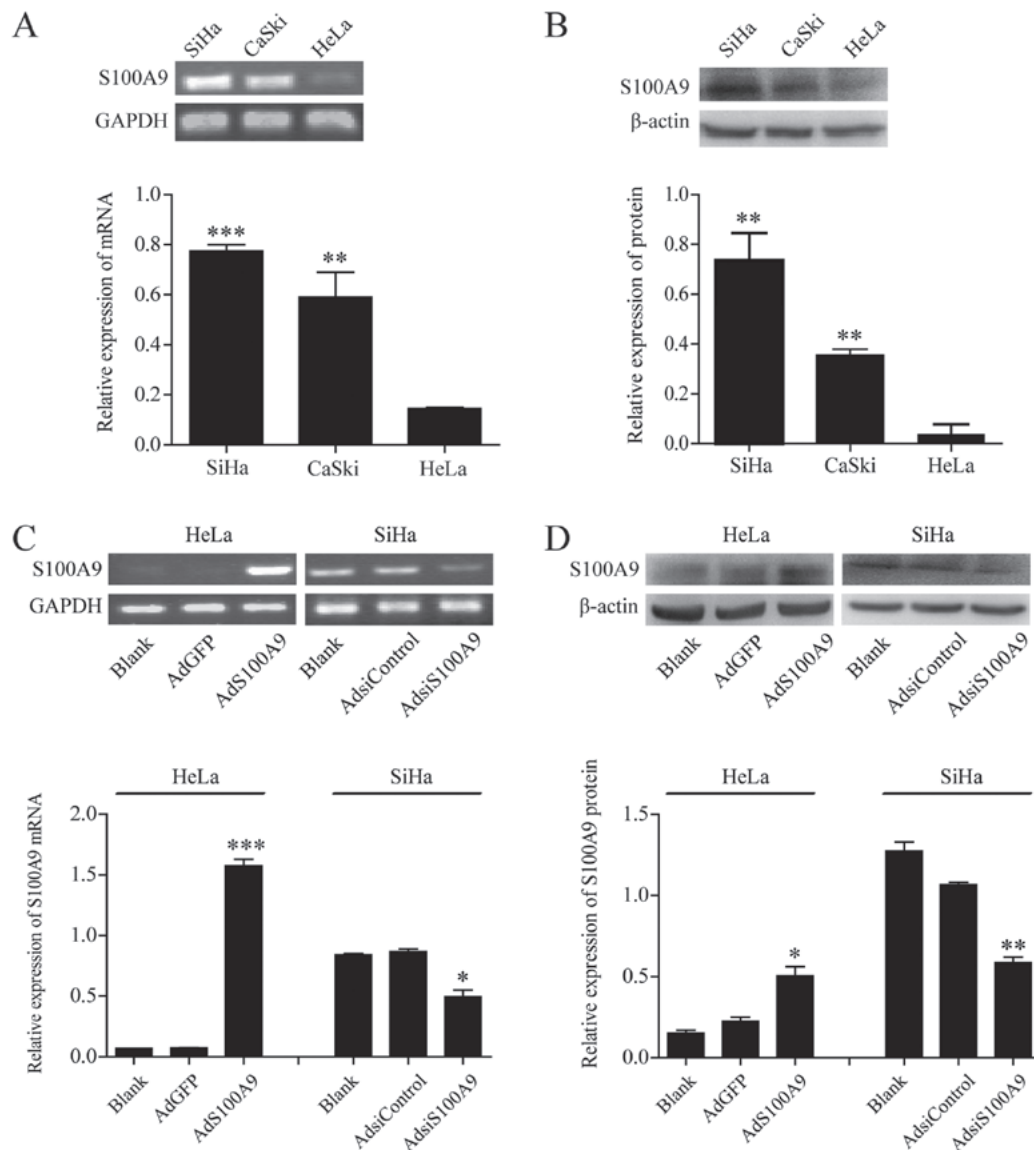


Figure 1. S100A9 is successfully overexpressed in HeLa cells and downregulated in SiHa cells via adenoviral infection. (A) mRNA expression of endogenous S100A9 in SiHa, CaSki and HeLa cell lines was detected by semi-quantitative PCR analysis. \*\*\* $P \leq 0.001$ , HeLa vs. SiHa and \*\* $P \leq 0.01$ , HeLa vs. CaSki. (B) Protein expression of endogenous S100A9 in SiHa, CaSki and HeLa cell lines was detected by western blot analysis. \*\* $P \leq 0.01$ , HeLa vs. SiHa and CaSki. HeLa cells were infected with AdGFP and AdS100A9 for 48 h, whereas SiHa cells were infected with AdSiControl and AdSiS100A9 for 48 h. The mRNA and protein expression of S100A9 in cervical carcinoma cell lines was detected by (C) semi-quantitative PCR and (D) western blot analysis. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \*\*\* $P \leq 0.001$ , \*\* $P \leq 0.01$  and \* $P \leq 0.05$ , AdS100A9 vs. AdGFP and AdSiControl. No significant difference between the AdGFP/AdSiControl and Blank groups of HeLa and SiHa cell lines were observed ( $P > 0.05$ ). Ad, adenovirus; Adsi, adenovirus containing small interfering RNA; GFP, green fluorescent protein; PCR, polymerase chain reaction.

**Statistical analysis.** All data are presented as the mean  $\pm$  standard deviation. Differences were analyzed using one-way ANOVA followed by a Tukey's multiple comparison test. All statistical analyses were performed using SPSS statistical package 17.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc.).  $P \leq 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression of endogenous S100A9 in cervical cancer cells.** We first evaluated the endogenous expression of S100A9 in SiHa, CaSki, and HeLa cell lines using semi-quantitative PCR and western blot analysis. As shown in Fig. 1A, the mRNA

expression levels of S100A9 in HeLa cells were significantly reduced than in SiHa and CaSki cells ( $P \leq 0.001$  and  $P \leq 0.01$ ). These results were confirmed by western blot analysis ( $P \leq 0.01$ ; Fig. 1B). Subsequently, semi-quantitative PCR and western blot analysis (Fig. 1C and D) were performed to detect the expression of S100A9 in HeLa and SiHa cells infected with AdS100A9 and AdSiS100A9 for 48 h, respectively. The expression of S100A9 observed in the AdS100A9 group was significantly increased compared with the AdGFP and Blank groups in HeLa cells ( $P \leq 0.001$  and  $P \leq 0.05$ ), whereas a significant decrease in S100A9 expression was detected in the AdSiS100A9 group compared with AdSiControl and Blank groups in SiHa cells ( $P \leq 0.05$  and  $P \leq 0.01$ ). These results indicated that S100A9 was successfully overexpressed in



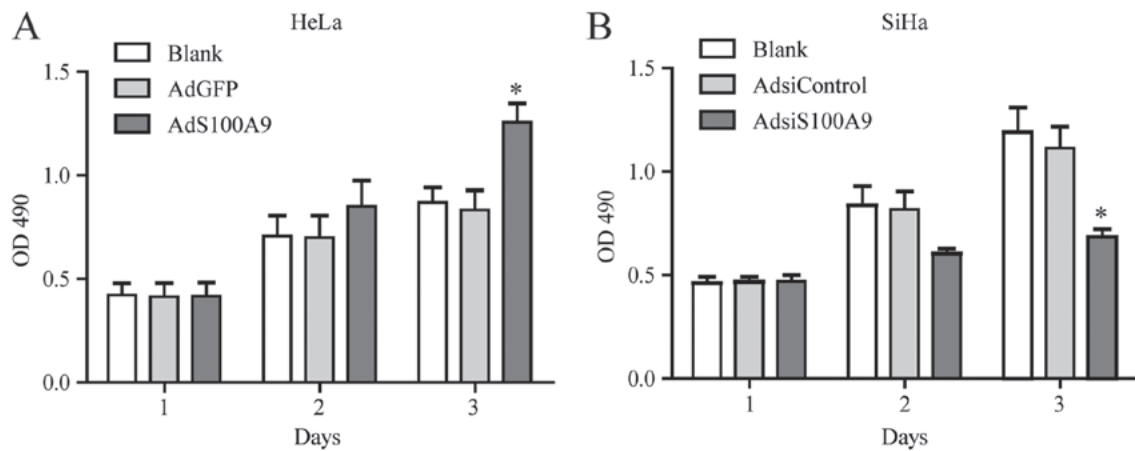


Figure 2. S100A9 upregulates the proliferation of cervical cancer cells. The proliferation of cervical cancer cells was determined by an MTT assay. (A) HeLa cells were infected with AdGFP or AdS100A9 for 3 days. (B) SiHa cells were infected with AdsiControl or AdsiS100A9 for 3 days. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P \leq 0.05$ , AdS100A9 vs. GFP and AdsiControl. Ad, adenovirus; Adsi, adenovirus containing small interfering RNA; GFP, green fluorescent protein.

HeLa cells and downregulated in SiHa cells via infection with AdS100A9 and AdsiS100A9, respectively.

#### *S100A9 promotes the proliferation of cervical cancer cells.*

To investigate the effects of S100A9 on the proliferation of cervical cancer cells, we treated the cells with AdS100A9 and AdsiS100A9 for 3 days and examined by an MTT assay (Fig. 2). Compared with the control groups, the proliferation of HeLa cells was significantly enhanced in the S100A9-overexpressing group on day 3 ( $P \leq 0.05$ ; Fig. 2A). On the contrary, the proliferation of SiHa cells was significantly inhibited in the S100A9-knockdown group compared with the control ( $P \leq 0.05$ ; Fig. 2B). These results demonstrated that overexpressed S100A9 could promote the proliferative potential of cervical cancer cells.

*S100A9 promotes the migration of cervical cancer cells.* We further studied the role of S100A9 in the migration of cervical cancer cells by wound healing and Transwell migration assays. The results revealed that the wound closure rate of S100A9-overexpressing HeLa cells was notably increased than the control group, whereas that of SiHa cells was impaired following knockdown of S100A9 (Fig. 3A). Similar results were observed in the Transwell migration assay. The number of HeLa cells migrated across the membrane in AdS100A9 group was significantly higher than that in the AdGFP group ( $P \leq 0.05$ ; Fig. 3B). These results are consistent with the wound healing ability of SiHa cells. The number of S100A9-depleted SiHa cells that migrated across the membrane was significantly lower compared with the control ( $P \leq 0.01$ ). Collectively, these results suggested that upregulated S100A9 expression may facilitate the migration of cervical cancer cells.

*S100A9 induces EMT in cervical cancer cells.* Emerging evidences support that EMT has a crucial role in the metastasis of primary tumors. To evaluate whether S100A9 promoted EMT in cervical cancer cells, western blot and immunofluorescence analyses were conducted to detect the levels of E-cadherin and vimentin. As presented in Fig. 4A,

overexpression of S100A9 in HeLa cells significantly decreased the expression of E-cadherin ( $P \leq 0.05$ ), and increased that of vimentin ( $P \leq 0.01$ ). On the contrary, knockdown of S100A9 in SiHa cells significantly decreased the expression of vimentin ( $P \leq 0.01$ ), and increased that of E-cadherin ( $P \leq 0.05$ ). These results were further confirmed by immunofluorescence assays (Fig. 4B). These findings suggested that S100A9 is involved in the regulation of EMT-related protein expression, and may act as a predictive factor and therapeutic target for preventing metastasis in cervical cancer.

#### *S100A9 promotes the activation of the Wnt/ $\beta$ -catenin signaling pathway in cervical cancer cells.*

It is well known that aberrant activation of the Wnt/ $\beta$ -catenin pathway serves a critical role in the development of cervical cancer. To investigate whether S100A9 was involved in the regulation of Wnt/ $\beta$ -catenin pathway in cervical cancer cells, western blot and semi-quantitative PCR analyses were performed to study the effects of S100A9 on the expression of  $\beta$ -catenin protein, and the transcriptional levels of c-Myc, Snail, and twist-related protein-1 (Twist), which are the classical target genes of Wnt/ $\beta$ -catenin pathway. As shown in Fig. 5A, after infecting HeLa cells with AdS100A9, overexpression of S100A9 significantly enhanced the accumulation of total  $\beta$ -catenin and nuclear  $\beta$ -catenin ( $P \leq 0.01$ ), but knockdown of S100A9 via the infection of SiHa cells with AdsiS100A9 decreased the accumulation of total  $\beta$ -catenin and nuclear  $\beta$ -catenin compared with the control groups ( $P \leq 0.001$  and  $P \leq 0.05$ ). Additionally, overexpression of S100A9 caused by infecting HeLa cells with AdS100A9 significantly promoted the transcription of c-Myc ( $P \leq 0.05$ ), Snail ( $P \leq 0.01$ ), and Twist ( $P \leq 0.05$ ) compared with the control. Conversely, the mRNA levels of the aforementioned target genes of the Wnt/ $\beta$ -catenin signaling pathway were significantly reduced when SiHa cells were infected with AdsiS100A9 compared with the control ( $P \leq 0.01$ ,  $P \leq 0.01$  and  $P \leq 0.001$ , respectively; Fig. 5B). These results demonstrated that S100A9-induced upregulation of c-Myc, Snail, and Twist expression in cervical cancer cell lines may be associated with the accumulation of total  $\beta$ -catenin and nuclear  $\beta$ -catenin.

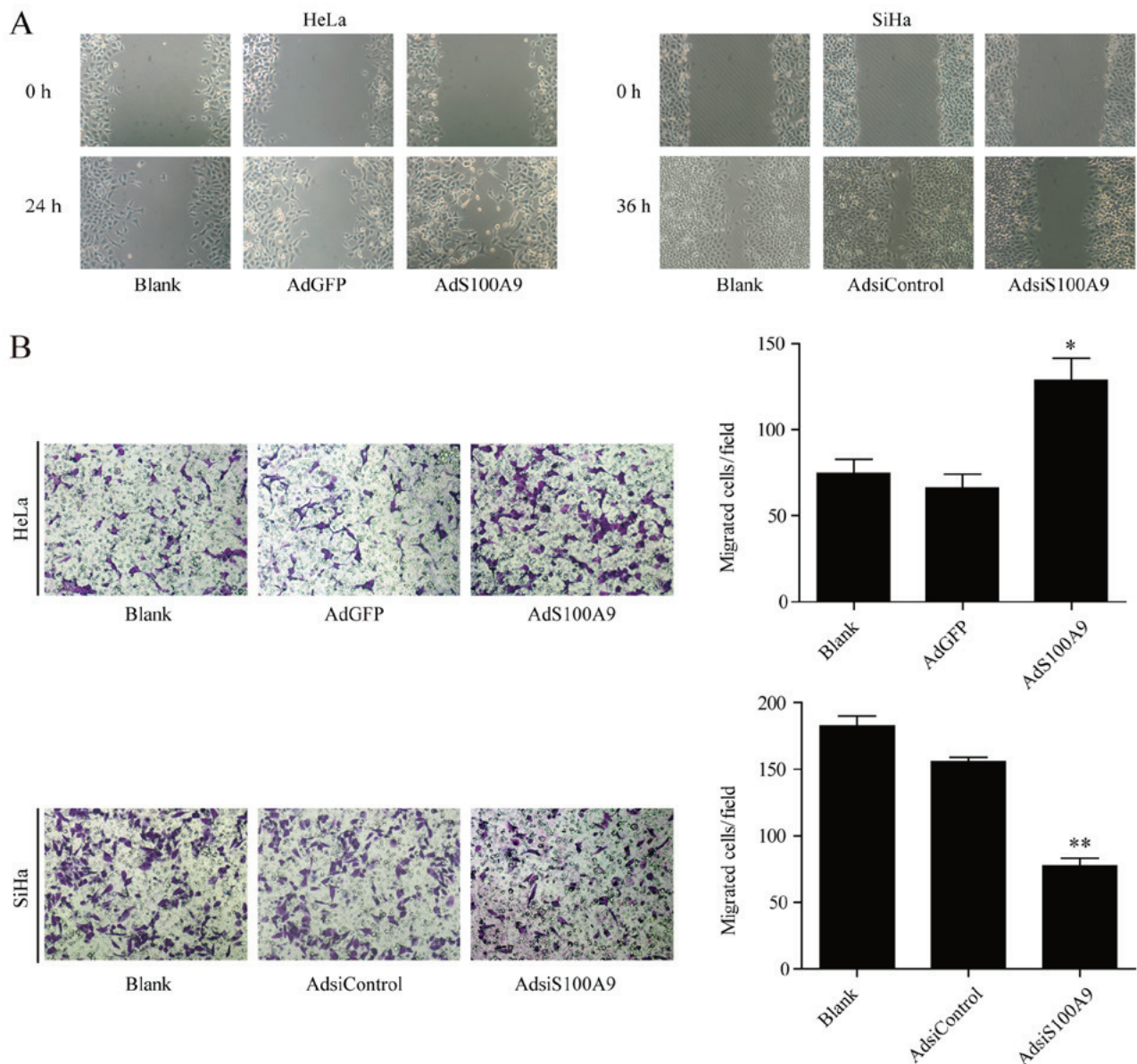


Figure 3. S100A9 promotes the migration of cervical cancer cells. (A) HeLa and SiHa cells were infected with AdS100A9 and AdsiS100A9, respectively, and the migration ability was determined by wound healing assay. The wound width was measured. (B) Transwell migration assay for analyzing the migration of HeLa and SiHa cells that were infected with AdS100A9 or AdsiS100A9 for 48 h. Representative images of transmembrane cells are shown. Data are presented as the mean  $\pm$  standard deviation of three individual measurements. \* $P \leq 0.05$ , AdS100A9 vs. AdGFP group; \*\* $P \leq 0.01$ , AdsiS100A9 vs. AdsiControl group. Ad, adenovirus; Adsi, adenovirus containing small interfering RNA; GFP, green fluorescent protein.

*S100A9-induced promotion of proliferation, migration and EMT in cervical cancer cells via activation of the Wnt/ $\beta$ -catenin pathway.* To determine whether Wnt/ $\beta$ -catenin pathway is involved in S100A9-induced promotion of proliferation, migration and EMT in cervical cancer cells, adenovirus Adsi $\beta$ -catenin was used as the antagonist to impair the activation of the Wnt/ $\beta$ -catenin pathway. As presented in Fig. 6A, the effects of S100A9 on the proliferation of HeLa cells was investigated by an MTT assay. After the treatment of cells with GST-hS100A9 at 10 and 20  $\mu\text{g/ml}$  for 72 h, the proliferation of HeLa cells was significantly increased than that of the control groups ( $P \leq 0.05$ ). Additionally, the results further showed a more notable effect in response to high concentration recombinant protein; thus, we selected 20  $\mu\text{g/ml}$  of recombinant protein for the following experiments. As

shown in Fig. 6B,  $\beta$ -catenin expression was significantly downregulated by adenovirus Adsi $\beta$ -catenin in HeLa cells compared with the control ( $P \leq 0.05$ ). After treatment with GST-hS100A9 and adenovirus Adsi $\beta$ -catenin, the mRNA levels of Snail, and Twist were significantly reduced in HeLa cells compared with the control groups ( $P \leq 0.05$  and  $P \leq 0.01$ ; Fig. 6C). In addition, treatment with Adsi $\beta$ -catenin significantly inhibited the proliferation and migration of HeLa cells induced by GST-hS100A9 ( $P \leq 0.05$  and  $P \leq 0.01$ ; Fig. 6D and E). Furthermore, the expression of EMT markers was examined in HeLa cells after treatment with and without AdRFP, Adsi $\beta$ -catenin, GST and GST-hS100A9. We observed that downregulation of  $\beta$ -catenin significantly suppressed the effects of S100A9 on the expression of E-cadherin and vimentin compared with the control groups ( $P \leq 0.05$  and

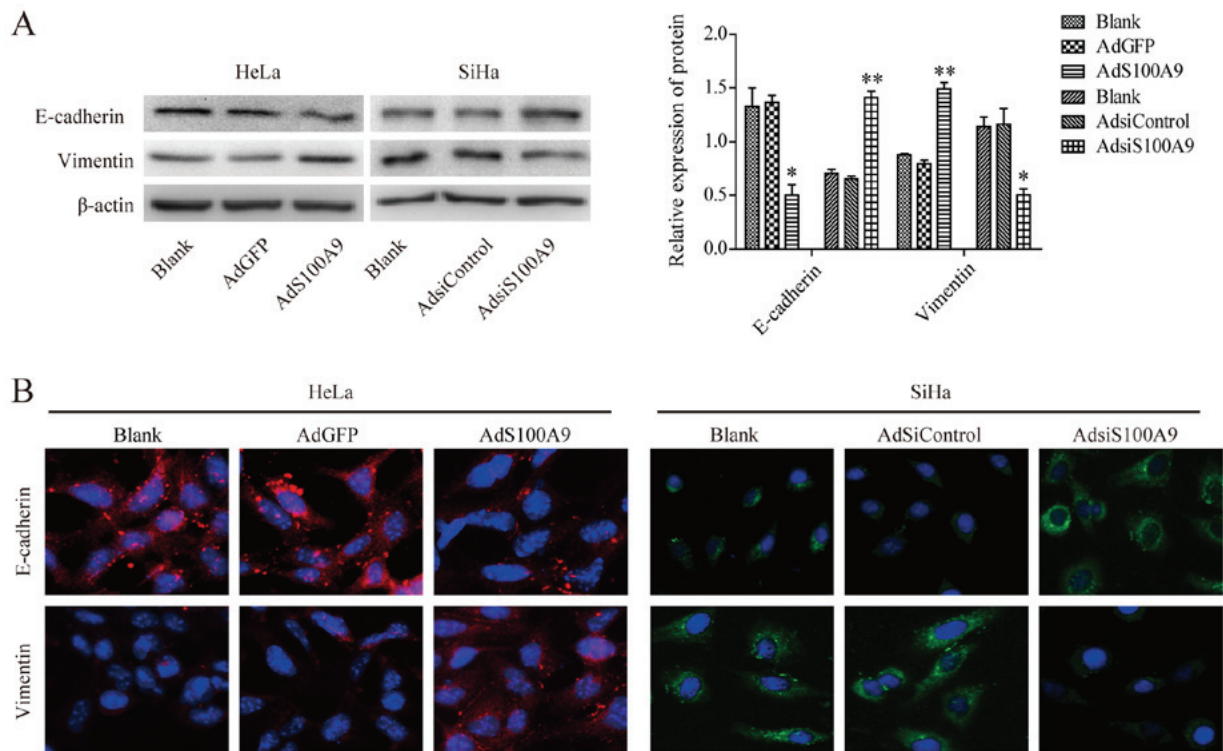


Figure 4. S100A9 promotes EMT in cervical cancer cells. After HeLa and SiHa cells were infected with AdS100A9 and AdSiS100A9, respectively, (A) the expression of EMT markers was detected by western blot analysis. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , AdS100A9 vs. AdGFP and AdSiControl. Data are presented as the mean  $\pm$  standard deviation of three individual measurements. (B) The levels of EMT markers were detected by immunofluorescence assays. The representative images are presented. Ad, adenovirus; Adsi, adenovirus containing small interfering RNA; EMT, epithelial-mesenchymal transition; GFP, green fluorescent protein.

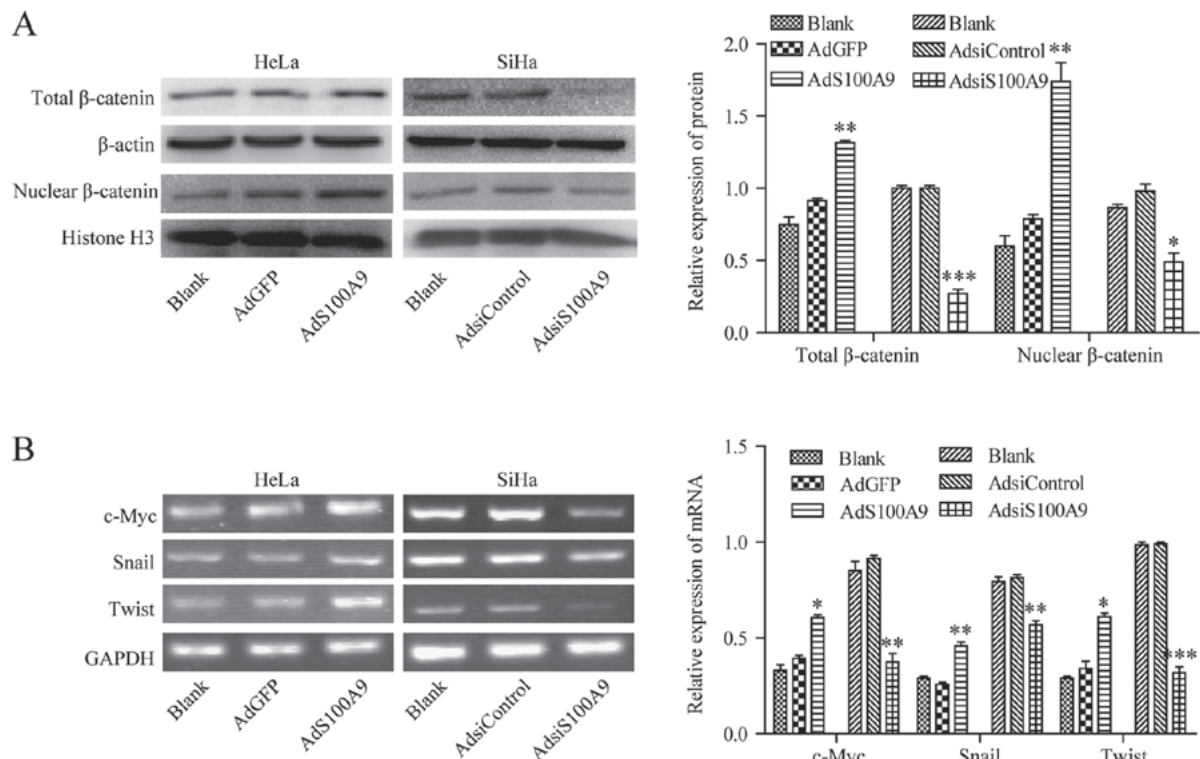


Figure 5. S100A9 activates the Wnt/ $\beta$ -catenin signaling pathway in cervical cancer cells. (A) After HeLa and SiHa cells were infected with AdS100A9 or AdSiS100A9, the accumulation of total and nuclear  $\beta$ -catenin was measured by western blot analysis.  $\beta$ -actin and histone were used as internal reference controls. \*\* $P \leq 0.01$ , AdS100A9 vs. AdGFP; \*\*\* $P \leq 0.001$ , \* $P \leq 0.05$ , AdSiS100A9 vs. AdSiControl. (B) After infecting HeLa and SiHa cells with AdS100A9 or AdSiS100A9, the mRNA expression of c-Myc, Snail, and Twist was detected using semi-quantitative polymerase chain reaction. GAPDH was used as an internal reference control. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , AdS100A9 vs. AdGFP and AdSiControl. Data are presented as mean  $\pm$  standard deviation of three individual measurements. Ad, adenovirus; Adsi, adenovirus containing small interfering RNA; Twist, twist-related protein-1.



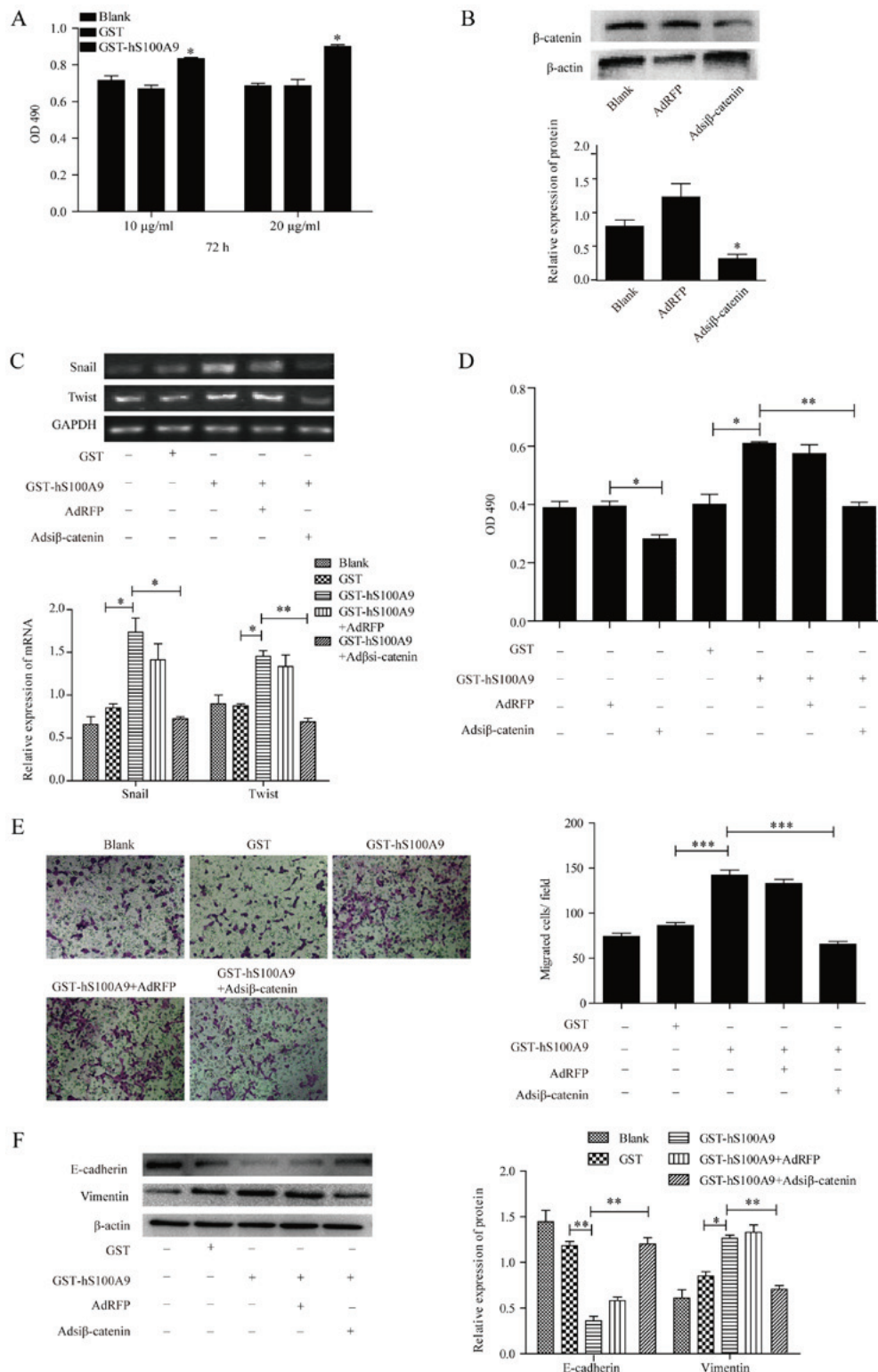


Figure 6. Underlying molecular mechanisms of S100A9-induced promotion of proliferation, migration and EMT in cervical cancer cells. (A) After treatment with or without GST-hS100A9 at different concentrations for 72 h, the proliferation of HeLa cells was measured by an MTT assay. \* $P \leq 0.05$ , GST-hS100A9 vs. GST control. (B) Adsiβ-catenin-mediated β-catenin knockdown in HeLa cells. After infection with Adsiβ-catenin, the expression of β-catenin was analyzed by western blot analysis. β-actin was used as internal reference control. \* $P \leq 0.05$ , Adsiβ-catenin vs. AdRFP. After infection with Adsiβ-catenin for 24 h, the infected cells were treated with or without GST-hS100A9 at 20 µg/ml for 48 h. The mRNA levels of Snail, and Twist were measured by (C) semi-quantitative polymerase chain reaction and the proliferation of HeLa cells was measured by (D) an MTT assay. GAPDH was used as an internal reference control. \* $P \leq 0.05$ , GST vs. GST-hS100A9; \*\* $P \leq 0.01$ , GST-hS100A9 vs. GST-hS100A9 + Adsiβ-catenin. (E) Transwell migration assay for analyzing the migration of HeLa cells that were infected with Adsiβ-catenin for 24 h and treated with or without GST-hS100A9 at 20 µg/ml for 48 h. Representative images of transmembrane cells are shown. The mean of transmembrane cells  $\pm$  standard deviation per microscopic field of three independent experiments was calculated. Magnification,  $\times 100$ . \*\*\* $P \leq 0.001$ , GST vs. GST-hS100A9, GST-hS100A9 + AdRFP vs. GST-hS100A9 + Adsiβ-catenin. (F) Western blot for analyzing the expression of EMT markers of HeLa cells that were infected with Adsiβ-catenin for 24 h and treated with and without GST-hS100A9 at 20 µg/ml for 48 h. β-actin was used as internal reference control. Data are presented as mean  $\pm$  standard deviation of three individual measurements. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , GST vs. GST-hS100A9, GST-hS100A9 + AdRFP vs. GST-hS100A9 + Adsiβ-catenin. Ad, adenovirus; Adsi, adenovirus containing small interfering RNA; EMT, epithelial-mesenchymal transition; GST, glutathione S-transferase; OD, optical density; RFP, red fluorescent protein.



$P \leq 0.01$ ; Fig. 6F). These findings demonstrated that S100A9 induced promotion of proliferation, migration and EMT through activation of Wnt/ $\beta$ -catenin pathway in cervical cancer cells.

## Discussion

Increasing evidence has indicated that the enhanced motility and invasiveness of cancer cells are associated with the progression of cervical cancer (14-16). In the present study, we observed that overexpression of S100A9 promoted the proliferation and migration of cervical cancer cells, eventually facilitating EMT, which was regulated by the Wnt/ $\beta$ -catenin signaling pathway. These findings suggested that S100A9 may be an important carcinogenic factor in the occurrence and progression of cervical cancer and may serve as an attractive therapeutic target for cervical cancer driven by various signaling pathways.

S100 proteins participate in a broad range of intracellular and extracellular functions by regulating calcium balance, cell apoptosis, migration, proliferation, differentiation, energy metabolism and inflammation (17). Overexpressed S100 proteins, including S100A9, were detected in a variety of cancers, including pancreatic, liver, and colorectal carcinomas (4,13,18). Compared with adjacent normal cervical tissues, increased levels of S100A9 were detected in squamous cervical cancer by two-dimensional gel electrophoresis followed by mass spectrometry, and the expression of S100A9 and RAGE in squamous cervical cancer was closely associated with histological differentiation and the tumor progression (6). Furthermore, various studies have shown that S100A9 could promote the proliferation and migration of different cell types, such as fibroblasts (19), MCF-7, MDA-MB-231, SH-EP, Kelly (20), HepG2 (4) and PNT1A cells (21). In accordance with these studies, our results suggested that overexpression of S100A9 enhanced the proliferation and migration properties of HeLa and SiHa cells.

EMT is required for several complex differentiation processes, including embryonic development, wound healing and tumor progression (22,23). Interestingly, Li *et al* (24) reported that S100A6 could facilitate the metastatic ability and EMT of cervical cancer cells, which was mediated by activating the PI3K/Akt signaling pathway. Additionally, S100A14 was determined to be a mediator of EMT that regulated the proliferation, migration and invasion of human cervical cancer cells (25). Based on these findings, we propose that overexpression of S100A9 resulted in a decrease in E-cadherin and an increase in vimentin expression in cervical cancer cells. Conversely, knockdown of S100A9 exhibited an antagonistic effect on the regulation of E-Cadherin and vimentin. These results suggested that S100A9 could enhance the mesenchymal properties of cervical cancer cells, which may be attributed to the induction of EMT.

The pivotal role of Wnt/ $\beta$ -catenin signaling pathway in tumor progression has been generally accepted, and cervical cancer has been linked with the aberrant activation of the Wnt/ $\beta$ -catenin pathway (22,26). In the present study, we reported that S100A9 enhanced the accumulation of  $\beta$ -catenin, and upregulated the mRNA expression of the target genes c-Myc, Snail, and Twist in cervical cancer cells. The results suggested

that S100A9 may exert significant effects on the induction of Wnt/ $\beta$ -catenin pathway in cervical cancer cells. In addition, previous studies have shown that  $\beta$ -catenin upregulation was involved in the proliferation and migration of cancer cells (27,28). Our results revealed that Wnt/ $\beta$ -catenin pathway was responsible for S100A9-induced promotion cervical cancer cell proliferation and migration, which could be partially suppressed by downregulation of  $\beta$ -catenin. Furthermore, we observed that activation of the Wnt/ $\beta$ -catenin pathway may be responsible for S100A9-induced EMT of cervical cancer cells, which could be partially suppressed by downregulating  $\beta$ -catenin. These findings suggested that S100A9 could induce EMT in cervical cancer cells via the Wnt/ $\beta$ -catenin signaling pathway.

Collectively, the present study revealed that S100A9-induced increases in cervical cancer cell proliferation and migration may be attributed to the promotion of EMT and aberrant activation of the Wnt/ $\beta$ -catenin signaling pathway. Additionally, S100A9 may be considered as a novel molecular target for the treatment of cervical cancer in future.

## Acknowledgements

We would like to thank T.C He (Medical Center, The University of Chicago) for the kind provision of the AdGFP, AdRFP, AdS100A9, Adsi $\beta$ -catenin recombinant adenoviruses, pGST-moluc and pGST-moluc-hS100A9 plasmids.

## Funding

This work was supported by National Natural Science Foundation grants of China (grant no. 81760475).

## Availability of data and materials

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

## Authors' contributions

HZ and XL performed most experiments and were major contributors in writing the manuscript. SY, HL, AL, YG, JZ and JX prepared experimental materials and reviewed the article. HS and LD made substantial contributions to the design of the study, and drafted the manuscript and revising it critically for important intellectual content. LZ gave final approval of the version to be published. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interest.

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