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Adenovirus vector-mediated upregulation of spermidine/spermine N^1-acetyltransferase impairs human gastric cancer growth *in vitro* and *in vivo*

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Spermidine/spermine N^1-acetyltransferase (SSAT) is the rate-limiting step in polyamine catabolism. In a previous study, we constructed a recombinant adenovirus, Ad-SSAT, which can express human SSAT. In the present study, we investigated the effect of upregulated and downregulated SSAT on gastric cancer cells. We found that upregulated SSAT could inhibit the growth of MGC803 and SGC7901 cells, whereas adverse results were found with downregulated SSAT. We further analyzed cell cycle profiles and the expression levels of the major cell cycle regulatory proteins of S phase. The results showed that the growth inhibition was caused by S phase arrest. Ad-SSAT suppressed the expression of cyclin A and nuclear factor E2F1 in MGC803 and SGC7901 cells. We observed the E2F promoter activity caused by Ad-SSAT using a reporter gene assay. We also investigated the antitumorigenicity of upregulated SSAT by Ad-SSAT using a SGC7901 xenograft model in nude mice. Our results suggest that the upregulation of SSAT by Ad-SSAT infection inhibited the growth of gastric cancer *in vitro* and *in vivo*. Ad-SSAT arrested gastric cancer cells in S phase, which was mediated through downregulation of the cyclin A-E2F signaling pathway. (*Cancer Sci* 2009; 100: 2126–2132)

Polyamines consist of putrescine, spermidine, and spermine. They are essential for cell growth and proliferation. Polyamine contents are often elevated in rodent and human neoplastic cells and tissues, compared with relevant normal cells and tissues.[1] Spermidine/spermine N^1-acetyltransferase (SSAT) is the rate-limiting enzyme in polyamine catabolism, which adds acetyl groups to the aminopropyl ends of spermidine and spermine.[2] SSAT is thought to prevent accumulation of the higher polyamines from becoming toxic to the cell, and so maintains a balanced ratio of intracellular polyamines according to the cellular needs. SSAT activity is highly regulated and induced rapidly in response to high intracellular levels of natural polyamines, hormones, physiological stimuli, drugs, and toxic agents.[3,4]

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death, with 700 000 deaths annually.[5] Studies have demonstrated a correlation between gastric cancer and elevated polyamine contents.[1] Our previous research demonstrated that the expression of ODC and AdoMetDC was increased in colorectal cancer tissue,[6,7] and downregulation of Ornithine Decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) could inhibit colorectal cancer growth.[8–10] However, this might be more effectively achieved by the superinduction of SSAT.[11] Therefore, we constructed a replication-deficient recombinant adenovirus (Ad) that could express human SSAT (Ad-SSAT) [12] and examined the inhibitory effect of Ad-SSAT on the growth of gastric cancer cells. Gene silencing is a fundamental mechanism of gene regulation, and RNAi is a powerful tool for gene silencing. Here we constructed a shRNA expression vector, pGPU6/GFP/Neo-shSSAT, to contrast the inhibitory effect of Ad-SSAT. We further evaluated the effects of Ad-SSAT on cell cycle distribution and investigated the underlying regulatory responses. We also investigated the *in vivo* growth-inhibitory effect using a SGC7901 xenograft model in nude mice. Our findings indicate that Ad-SSAT could significantly suppress the growth of gastric cancer, induce S phase arrest of the gastric cancer cells, and suppress the expression of cyclin A and E2F1. We presume that Ad-SSAT inhibits cyclin A through the downregulation of nuclear factor E2F1.

Materials and Methods

Cells and reagents. The human gastric cancer cell lines MGC803 and SGC7901, maintained in our laboratory, were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2. The 3-C(4,5-dimethylthiaol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(-4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)/phenazine methosulfate (PMS) kit was purchased from Promega (Madison, WI, USA). The polyamine standards (putrescine, spermidine, and spermine) and densyl chloride for HPLC were purchased from Sigma (St Louis, MO, USA). The β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the ECL western blotting detection system was from Millipore (Bedford, MA, USA). A mouse anti-β-actin monoclonal antibody was purchased from Santa Cruz Biotechnology. Rabbit antihuman polyclonal antibodies against cyclin A, cyclin-dependent kinase (Cdk)2, and the E2F-1 protein were purchased from Neomarkers (Fremont, CA, USA). The plasmid pGL3-E2F was a gift from Professor Yho Takuwa (Kanazawa University School of Medicine).[13] Male nude mice aged 5 weeks were obtained from Shanghai Slac Laboratory Animal Co. (Shanghai, China) and fed a standard rodent diet under specific pathogen-free conditions.

Construction of pGPU6/GFP/Neo-shSSAT and transfection. The shRNA expression vector pGPU6/GFP/Neo-shSSAT was commercially obtained from GenePharma (Shanghai, China). The siRNA duplex was designed to SSAT (NM_002970). The sequences were as follows: 5′-GGA CAC AGC ATT GTT GGT T-3′ (sense) and 5′-AAC CAA CAA TGC TGT GTC C-3′ (antisense). The sequences of non-functional control siRNA were 5′-GTT CTC CGA ACG TGT CAC GT-3′ (sense) and 5′-ACG TGA CAC GTT CGG AGA A-3′ (antisense). Transfection of siRNA or plasmid was carried out using Lipofectamine 2000

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(Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Measurement of polyamine content. Polyamine content was measured by HPLC analysis. MGC803 and SGC7901 cells were infected with Ad-GFP, Ad-SSAT by 50 and 25 MOI, or transfected with pGPU6/GFP/Neo-NC and pGPU6/GFP/Neo-shSSAT. After 48 h, cells were trypsinized and washed twice with PBS. Intracellular polyamines were extracted from cell pellets with 10% trichloroacetic acid and then the polyamines were mixed with a two-fold volume of dansyl chloride and dansylated in the presence of sodium carbonate for 20 min at 70°C. Dansylated polyamines were quantified using reverse-phase HPLC.

Cell proliferation analysis. To observe the effect of the adenovirus and pGPU6/GFP/Neo-shSSAT on cell growth *in vitro*, the MTS assay was used to draw cell growth curves. The MGC803 and SGC7901 cells were seeded onto 96-well plates at 4000 cells/well, and cultured overnight. Then the cells were treated with PBS, Ad-GFP, and Ad-SSAT by 50 and 25 MOI, or transfected with pGPU6/GFP/Neo-NC and pGPU6/GFP/Neo-shSSAT. All experiments were carried out six times. After 24, 48, 72, 96, and 120 h, 20 μL MTS solution (with PMS) was added to each well and maintained for 2 h at 37°C. The optical density was measured at 490 nm. Cell viability was assessed by absorbance at 490 nm.

Colony-forming assay. The MGC803 and SGC7901 cells were infected with Ad-GFP, Ad-SSAT by 50 and 25 MOI, or transfected with pGPU6/GFP/Neo-NC and pGPU6/GFP/Neo-shSSAT. After 24 h, the cells were dissociated into single cells and seeded in six-well culture plates at 1000 cells/well in triplicate wells. After incubation for 14 days, the cells were stained with Giemsa for 20 min, and the numbers of colonies containing more than 50 cells were counted. The colony formation rate was calculated as the number of colonies/the number of cells planted.

Cell cycle analysis. Flow cytometric analysis was carried out to define the cell cycle distribution. MGC803 and SGC7901 were seeded at a density of 3×10^5 cells/well in six-well plates and infected with Ad-GFP or Ad-SSAT at MOI 50 and 25. After 72 h, the cells were harvested via trypsinization, washed with cold PBS, and processed for cell cycle analysis. Briefly, 2×10^5 cells were resuspended in 0.5 mL cold PBS, 1 mL 70% cold ethanol was added, and the cells were incubated for 1 h at 4°C. After centrifugation,the pallet was washed with cold PBS, suspended in 0.3 mL PBS, and incubated with 50 μL RNase (1 mg/mL) for 30 min at 37°C. The cells were kept on ice for 10 min and incubated with 500 μL propidium iodide (50 mg/L) for 30 min in the dark. The cell cycle distribution of the cells of each sample was then determined using FACScan (Becton-Dickinson, Franklin Lakes, NJ, USA).

Western blot analysis. After the MGC803 and SGC7901 cells were infected with Ad-GFP or Ad-SSAT at MOI 50 and 25 in RPMI-1640 medium containing 5% FBS for 72 h, they were collected with a cell scraper and washed three times with ice-cold PBS. Total cell lysates were prepared in an extraction buffer containing 0.05 M Tris (pH 8.0), 0.15 M NaCl, 0.02% sodium azide, 0.1% SDS, 100 mg/mL phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, and 1% nonionic detergent (NP)-40. The nuclear protein was prepared as previously described.[14] Sample protein concentrations were determined by bicinchoninic acid protein assay. The total protein (10–50 μg/lane) was electrophoresed, separated on 5% stacking and 12% resolving SDS-PAGE gels, and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA), which were blocked in 5% non-fat dry milk in Tris-buffered saline-Tween (TBST, pH7.6). The membrane was incubated overnight with the appropriate primary antibody, subsequently, the membrane was rinsed in TBST (pH 7.6) for 40 min, and incubated with a secondary antibody conjugated to horseradish peroxidase. The membranes were then reacted for 5 min with luminol substrate and exposed to X-ray film (AGFA, Mortsel, Belgium). The X-ray film was analyzed using light-density analysis software (Alpha Imager, Alpha Innotech Corporation, San Leandro, CA, USA). All protein levels were assessed by densitometry, with β-actin used as a control.

Transient transfection and luciferase activity assay. The MGC803 and SGC7901 cells were seeded in 24-well plates at a density of 1×10^5 cells/well prior to transfection. Transient transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, each well included 1.5×10^5 cells, 1 μg PGL3 construct, 0.04 μg internal control vector pRL-TK, 2 μL Lipofectamine 2000, and 50 μL RPMI-1640 media without serum and antibiotics. After 24 h, the cells were then infected with Ad-GFP at 50 MOI and Ad-SSAT at 25 MOI. At 72 h after transfection, the cells were harvested for the promoter activity assay. Then, the cells were rinsed twice with PBS buffer and cell lysates were prepared by manually scraping the cells from culture plates in the presence of 1× passive lysis buffer. Cell lysate (20 μL) was transferred into a luminometer tube containing 100 μL LAR II (Promega, Madison, WI, USA), and the firefly luciferase activity (M1) was measured first. The *Renilla* luciferase activity (M2) was then measured after adding 100 μL of Stop & Glo Reagent (Promega, Madison, WI, USA). The results were calculated and expressed as the ratio of M1/M2. Analyses were carried out two or three times with double replicates.

Tumor growth studies *in vivo*. The SGC7901 cells were infected with Ad-SSAT and Ad-GFP by 50 and 25 MOI for 48 h, harvested, washed three times with PBS, and in RPMI-1640 medium. The cell suspensions (2×10^6 cells) in a total volume of 100 μL were then injected subcutaneously into 5-week-old BALB/c nude mice. Tumor growth was monitored every 4 days starting from the eighth day after injection. Tumor volume was calculated with the following formula: volume (mm^3) = $ab^2/2$ with $b < a$.[15] On the 33rd day after inoculation, all mice were killed, and the tumor masses were weighed.

Statistical analysis. All data are presented as mean ± SD from three separate experiments. Student's *t*-test was used to compare the data, and $P < 0.05$ was considered statistically significant. All results were analyzed using SPSS (Chicago, IL, USA) 10.0 statistical software.

Results

Effect of Ad-SSAT and pGPU6/GFP/Neo-shSSAT on the SSAT gene expression in gastric cancer cells. Western blot analysis was carried out to detect the effect of replication-deficient Ad-SSAT infection and pGPU6/GFP/Neo-shSSAT transfection on intracellular SSAT protein levels. The SSAT levels in Ad-SSAT-infected cells were significantly higher than in cells infected with Ad-GFP or treated with PBS, whereas cells transfected with pGPU6/GFP/Neo-shSSAT could significantly block the expression of SSAT protein. The results analyzed using light-density analysis software (Alpha Imager) showed that SSAT expression in Ad-SSAT-infected MGC803 cells was 265% of the level in cells infected with Ad-GFP. The corresponding figure in SGC7901 cells was 210%. The SSAT expression in pGPU6/GFP/Neo-shSSAT-transfected MGC803 cells was 15% of the level in cells transfected with pGPU6/GFP/Neo-shNC, and the corresponding figure in SGC7901 cells was 27% (Fig. 1).

Effect of Ad-SSAT and pGPU6/GFP/Neo-shSSAT on polyamine content in gastric cancer cells. We next evaluated the polyamine concentration caused by the adenovirus and pGPU6/GFP/Neo-shSSAT. Polyamines in adenovirus-infected or -uninfected gastric cancer cells and in gastric cancer cells transfected with pGPU6/GFP/Neo-NC or pGPU6/GFP/Neo-shSSAT were separated by ion-pairing, reverse-phase HPLC. As shown in Table 1, upregulation of SSAT decreased the content of spermidine and spermine in MGC803 and SGC7901 cells, which was correlated

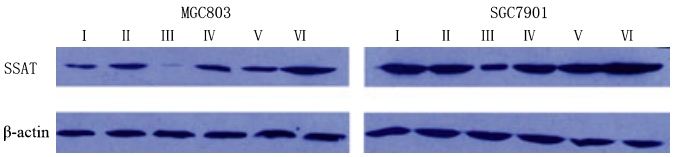

Fig. 1. Western blot analysis for spermidine/spermine N^1-acetyltransferase (SSAT) expression in MGC803 and SGC7901 cells. MGC803 and SGC7901 cells were transfected with pGPU6/GFP/Neo-shNC and pGPU6/GFP/Neo-shSSAT or infected with adenovirus by 50 and 25 MOI. I, mock; II, pGPU6/GFP/Neo-shNC-transfected; III, pGPU6/GFP/Neo-shSSAT-transfected; IV, mock; V, Ad-GFP-infected; VI, Ad-SSAT-infected.

Table 1. Effect of adenovirus (Ad)-spermidine/spermine N^1-acetyltransferase (SSAT) and pGPU6/GFP/Neo-shSSAT on polyamine content in MGC803 and SGC7901 cells (n = 3)

	Polyamine pools (pM/10^6cells)		
Cell lines and treatment	Putrescine	Spermidine	Spermine
MGC803	267 ± 33	9689 ± 987	10 987 ± 1242
+Ad-GFP	272 ± 29	9432 ± 931	10 559 ± 1179
+Ad-SSAT	283 ± 36	4981 ± 672*	5968 ± 716*
+pGPU6/GFP/ Neo-NC	291 ± 31	9219 ± 901	9956 ± 1023
+pGPU6/GFP/ Neo-shSSAT	297 ± 32	14 521 ± 1423**	15 624 ± 1487**
SGC7901	289 ± 34	7143 ± 671	8267 ± 812
+Ad-GFP	311 ± 35	6237 ± 737	7689 ± 698
+Ad-GFP	346 ± 43	2436 ± 401*	2981 ± 367*
+pGPU6/GFP/ Neo-NC	341 ± 32	7013 ± 702	7989 ± 703
+pGPU6/GFP/ Neo-shSSAT	352 ± 46	13 324 ± 1234**	13 997 ± 1301**

Data are mean ± SD. *P < 0.05 versus infection with Ad-GFP or uninfected cells; **P < 0.05 versus transfected with pGPU6/GFP/Neo-NC, pGPU6/GFP/Neo-shSSAT. n, refer to the times repeated in the experiment.

with the acceleration of polyamne catabolism. However, down-regulation of SSAT elevated the content of spermidine and spermine in MGC803 and SGC7901 cells.

Effect of Ad-SSAT and pGPU6/GFP/Neo-shSSAT on gastric cells. We examined the inhibitory effects of upregulated SSAT on the growth of MGC803 and SGC7901 cells *in vitro* using the MTS assay; we also used pGPU6/GFP/Neo-shSSAT to contrast the effect. As shown in the cell growth curves (Fig. 2), the expression of SSAT affected the growth of both MGC803 and SGC7901 cells. Ad-SSAT inhibited their proliferation, whereas the cells transfected with pGPU6/GFP/Neo-shSSAT grew faster compared with the control groups (treated with Ad-GFP, PBS, or transfected with GPU6/GFP/Neo-shNC).

In the colony-formation assay, the plating efficiencies in MGC803, Ad-GFP-infected MGC803, Ad-SSAT-infected MGC803, pGPU6/GFP/Neo-shNC-transfected MGC803, and pGPU6/GFP/Neo-shSSAT-transfected MGC803 were 43.2 ± 5.3%, 40.8 ± 4.6%, 11.2 ± 2.6%, 41.6 ± 5.1%, and 63.6 ± 8.2% respectively. The plating efficiencies in SGC7901, Ad-GFP-infected SGC7901, Ad-SSAT-infected SGC7901, pGPU6/GFP/Neo-shNC-transfected SGC7901, and pGPU6/GFP/Neo-shSSAT-transfected SGC7901 were 52.3 ± 6.2%, 51.1 ± 5.9%, 15.4 ± 2.3%, 51.9 ± 7.2%, and 65.2 ± 6.9% (Fig. 3). The results showed that the colony formation rate was significantly decreased in MGC803 and SGC7901 cells with upregulated SSAT and increased in MGC803 and SGC7901 cells with down-regulated SSAT compared with the control groups.

Upregulated SSAT arrests gastric cancer cells in S phase. We further analyzed the cell cycle profiles to examine the mechanism by which Ad-SSAT retards the growth of gastric cancer cells *in vitro*. The distribution of MGC803 and SGC7901 cells at different points of the cell cycle was analyzed using flow cytometry 72 h after infection. The results show that Ad-SSAT caused more MGC803 and SGC7901 cells to halt their progression through the cell cycle compared with the controls (P < 0.05) (Fig. 4; Table 2). MGC803 cells were arrested in S phase: 27 ± 2.3% in Ad-SSAT-infected cells, 18 ± 2.6% in PBS-treated cells, and 19 ± 2.1% in Ad-GFP-infected cells. In SGC7901 cells, Ad-SSAT treatments caused 31 ± 2.4% of the cells to arrest in S phase. There was no significant difference in cell cycle distribution between uninfected cells and cells infected with Ad-GFP (P > 0.05).

Ad-SSAT inhibits expression of cyclin A and E2F1. We further analyzed the correlation between the expression levels of the major cell cycle regulatory proteins of S phase and Ad-SSAT. The expression levels of cyclin A, cdk2, and E2F1 were detected by western blotting. The results (Fig. 5) showed that the levels of cyclin A and E2F1 protein decreased in the Ad-SSAT-infected cells compared with cells infected with Ad-GFP or treated with PBS. However, there were no obvious changes in the cdk2 protein levels in the three groups.

After demonstrating that Ad-SSAT suppressed the expression of cyclin A and E2F1, we further evaluated the effect of Ad-SSAT on the E2F promoter. The E2F promoter luciferase reporter plasmid was transfected into MGC803 and SGC7901 cells and a luciferase activity assay was carried out, as shown in Figure 6, where M1/M2 represents the relative luciferase activity. The results showed that E2F promoter activity decreased by ~80% in Ad-SSAT-infected MGC803 cells and ~60% in Ad-SSAT-infected SGC7901 cells compared with cells infected with Ad-GFP or treated with PBS. The results showed that Ad-SSAT inhibited E2F1 expression by suppressing the promoter activity.

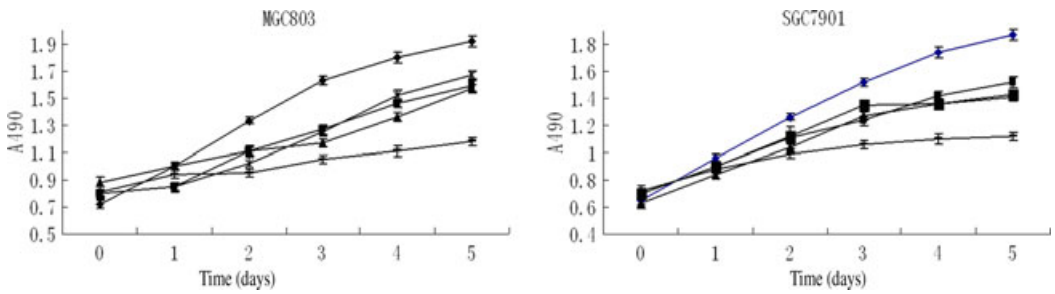

Fig. 2. Effect of adenovirus (Ad)-spermidine/spermine N_1-acetyltransferase (SSAT) and pGPU6/GFP/Neo-shSSAT on the growth of MGC803 and SGC7901 cells. MGC803 and SGC7901 cells were infected with Ad-GFP (▲), Ad-SSAT(-) at MOI 25 and 50, and transfected with pGPU6/GFP/Neo-shNC (×), pGPU6/GFP/Neo-shSSAT (◆), or treated with PBS (■). After 24 h of incubation, absorbance was measured every day for 5 days. Each bar represents the mean ± SD of five experiments.

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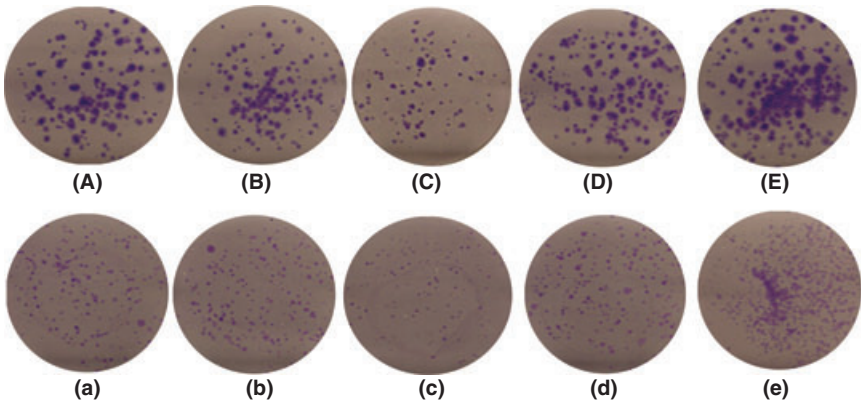

Fig. 3. Effect of adenovirus (Ad)-spermidine/spermine N^1-acetyltransferase (SSAT) on growth of MGC803 and SGC7901 cells. Cells were cultured in triplicate sets of six-well tissue culture plates with RPMI-1640+10% FBS for 14 days. Colonies were visualized by Giemsa staining. Colonies transfected with pGPU6/GFP/Neo-shSSAT increased, whereas Ad-SSAT-infected cells were significantly decreased compared to their two controls ($P < 0.05$). (A) MGC803; (B) Ad-GFP-infected MGC803; (C) Ad-SSAT-infected MGC803; (D) pGPU6/GFP/Neo-shNC transfected MGC803; (E) pGPU6/GFP/Neo-shSSAT-transfected MGC803; (a) SGC7901; (b) Ad-GFP-infected SGC7901; (c) Ad-SSAT-infected SGC7901; (d) pGPU6/GFP/Neo-shNC-transfected SGC7901; (e) pGPU6/GFP/Neo-shSSAT-transfected SGC7901.

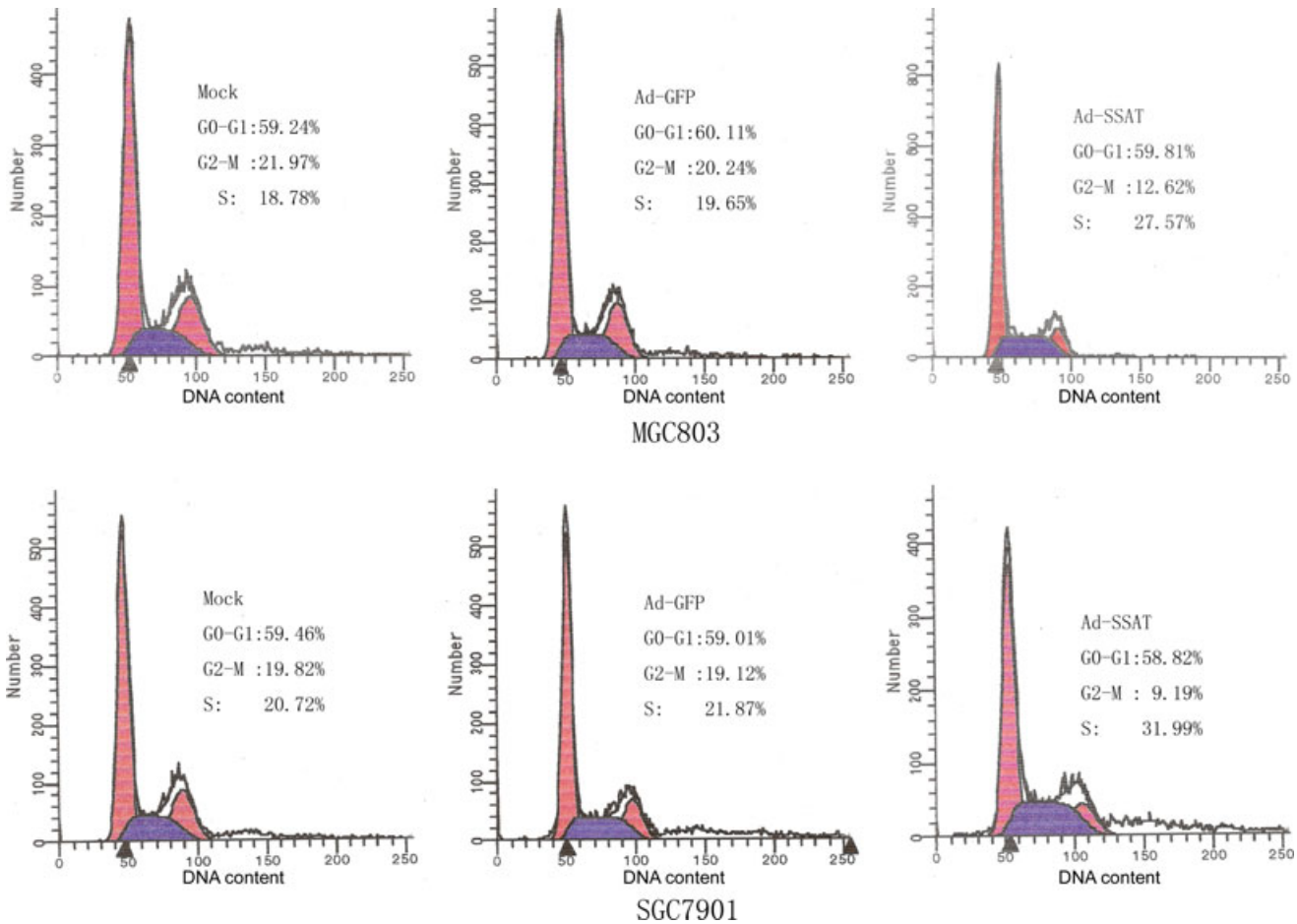

Fig. 4. Effects of adenovirus (Ad)-spermidine/spermine N^1-acetyltransferase (SSAT) on the cell cycle of MGC803 and SGC7901 cells. Cells were treated with 50 or 25 MOI of Ad-GFP, Ad-SSAT, or PBS (mock), then collected and dyed with propidium iodide for cell cycle analysis. The data are representative of three separate experiments.

Suppression of tumor growth *in vivo*. After examining the inhibitory effects of upregulated SSAT on the growth of MGC803 and SGC7901 cells *in vitro* we next investigated the antitumorigenicity of Ad-SSAT using a SGC7901 xenograft model in nude mice. As shown in Figure 7(c), visible tumors in the SGC7901 and SGC7901/GFP groups were readily detectable 8 days after implantation and grew rapidly in the next days. In contrast, SGC7901, the cell treated with Ad-SSAT cells remarkably delayed tumor growth, and visible tumors were only detectable by day 11 after implantation. These tumors of

Table 2. Distribution of MGC803 and SGC7901 cells in S phase of the cell cycle

Cell lines and treatment	Percent of total cells
	S ($\bar{X}$ ± SD)
MGC803 cell (control)	18 ± 2.6
+Ad-GFP	19 ± 2.1
+Ad-SSAT	27 ± 2.3*
SGC7901 cell (control)	20 ± 2.9
+Ad-GFP	21 ± 3.1
+Ad-SSAT	31 ± 2.4*

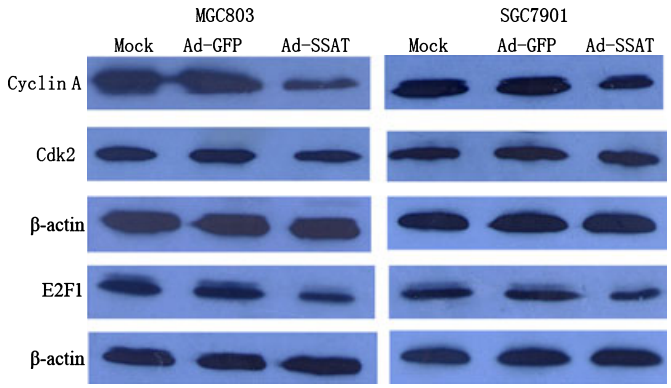

Fig. 5. Western blot analysis of Cyclin A, Cdk2, and E2F1 gene expression in MGC803 and SGC7901 cells. Total protein was extracted 3 days after infection with adenovirus (Ad)-spermidine/spermine N^1-acetyltransferase (SSAT) or the control vector. The blot was probed with either anti-cyclin A, anti-Cdk2, or anti-E2F1 antibodies. Equal loading was verified using an anti-β-actin antibody.

SSAT-upregulated cells remained at a similarly small average size of approximately 500 mm^3 by day 33 after implation.

On day 33 after tumor implantation, mice were killed and tumors were removed and weighed. In tumors from SSAT-upregulated groups, size and weight were significantly decreased by 68.3%, demonstrating an *in vivo* growth-inhibitory effect (Fig. 7).

Discussion

Polyamines are important molecules in cell proliferation and differentiation. It was not until 1968 that an association of increased polyamine synthesis with cell proliferation and cancer progression was established. Increases in polyamine concentrations and elevated polyamine synthesis activity were found in many tumors.[16] SSAT is the rate-limiting enzyme in polyamine catabolism. Although polyamines are required for cell growth and differentiation, SSAT is thought to prevent accumulation of the higher polyamines, to maintain a balanced ratio of intracellular polyamines. Resting SSAT levels are very low; its content is adjusted in response to alterations in polyamine content to maintain polyamine homeostasis. Additionally, SSAT activity can be induced by a number of stimuli, including polyamines, polyamine analogs, toxins, hormones, cytokines, non-steroidal anti-inflammatory agents, natural products, and stress pathways, and by ischemia-reperfusion injury.[2,17–19]

In the present study, western blotting demonstrated that Ad-SSAT significantly increased SSAT protein levels in MG803 and SGC7901 cells. Cell proliferation analysis and colony-forming assays showed that Ad-SSAT had a significant inhibitory effect on gastric cancer cell growth, whereas pGPU6/GFP/Neo-shSSAT made the cells grow faster. We also evaluated the antitumorigenicity of upregulated-SSAT and observed a notable *in vivo* growth-inhibitory effect. We know that polyamines are essential for cell growth and proliferation. Polyamine contents are often elevated in cancer cells and tissues. In our study, we measured the polyamine content using HPLC analysis. Ad-SSAT decreased the content of spermidine and spermine in MGC803 and SGC7901 cells. An increase in SSAT expression accelerated the polyamine catabolism, reduced polyamine content, and inhibited cell growth. However, induction of SSAT may have an antitumor effect not only by reducing polyamine content. High levels of SSAT cause a fall in acetyl-CoA and malonyl-CoA, which would decrease fatty acid synthesis. Drugs inhibiting acetyl-CoA carboxylase, which alters fatty acid synthesis, have antitumor effects.[20]

Furthermore, we detected the cell cycle profiles and the expression levels of cell cycle-related proteins. Flow cytometry showed that Ad-SSAT induced the S phase arrest of gastric cells. Cyclin A kinase, an enzyme required for coordinating S phase progression, forms stable *in vivo* complexes with E2F1, which binds to the retinoblastoma gene product and is involved in the timely activation of genes whose products contribute to G_1 exit and S phase traversal.[21] Cyclin A starts to accumulate during S phase and is abruptly destroyed before metaphase. The synthesis of cyclin A is mainly controlled at the transcription level, involving E2F and other transcription factors.[22] In our study, western blotting indicated that Ad-SSAT could decrease the protein level of cyclin A and E2F1.

E2F is a family of transcription factors involved in controlling proliferation, apoptosis, development, and tumorigenesis. Studies have shown that activation of E2F-dependent transcription promotes cell cycle progression and S phase entry. The E2F and the retinoblastoma protein (pRB) families of transcription factors play a pivotal role in cell division control.[23,24] E2F1 binds largely to the un(der)phosphorylated (G_1) form of RB (pRB).[25–27]

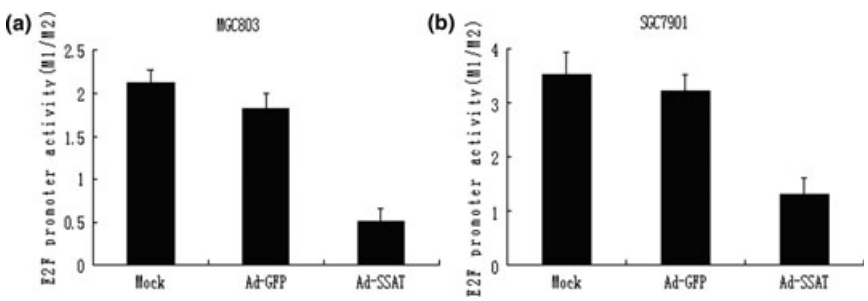

Fig. 6. E2F promoter activity in (a) MGC803 and (b) SGC7901 cells. pGL3-E2F and pRL-TK were cotransfected into MGC803 and SGC7901. At 24 h after transfection, cells were infected with adenovirus (Ad)-GFP and Ad-spermidine/spermine N^1-acetyltransferase (SSAT) for 72 h. E2F promoter activity was measured as described in Materials and methods. Each bar represents the mean ± SD of three experiments.

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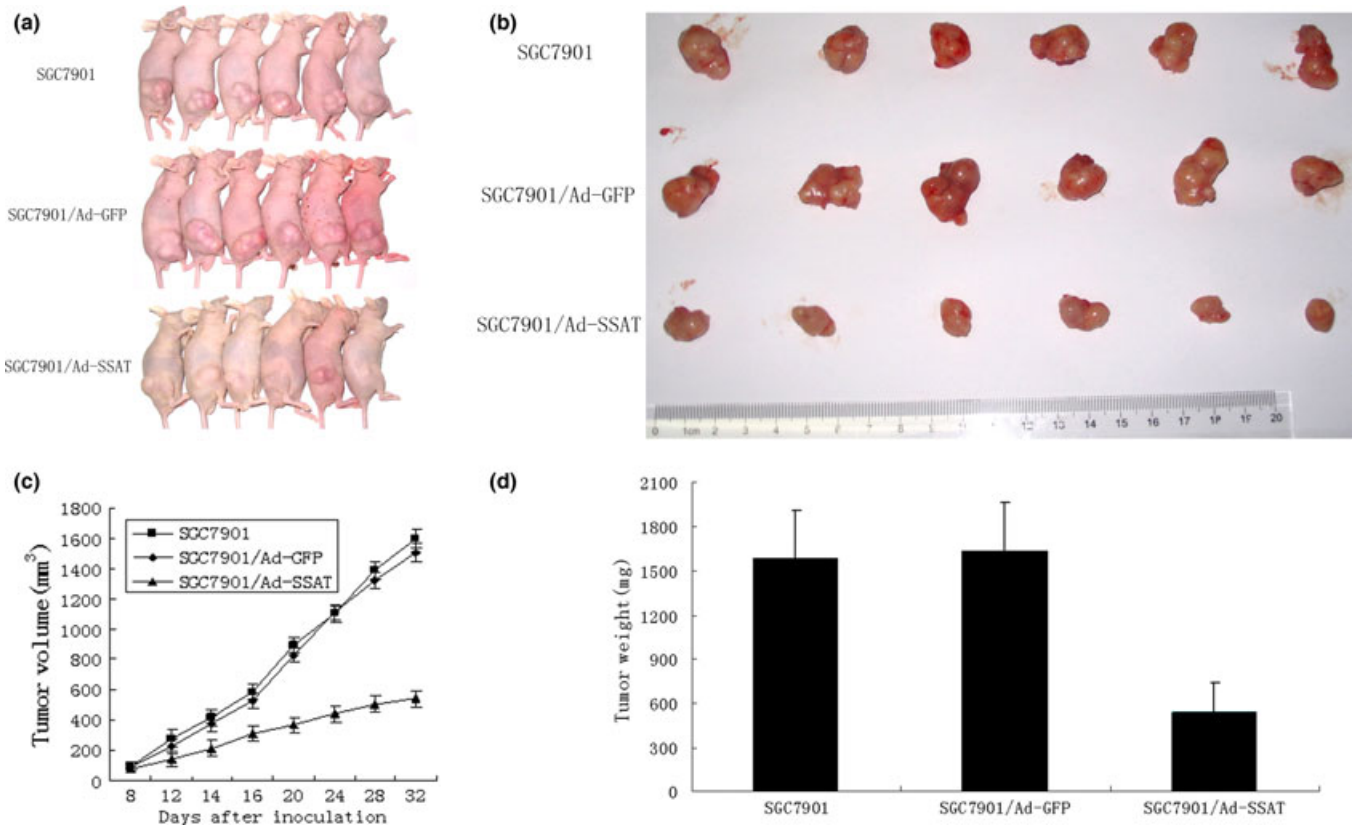

Fig. 7. Suppression of tumor growth *in vivo*. Approximately 2×10^6 tumor cells were implanted subcutaneously into the right flank area of each mouse. Tumor growth and animal weight were monitored every 4 days after the eighth day after injection (c) showed the time course of estimated mean tumor volume of either SGC/7901/SSAT (▲), SGC7901/GFP (◆), or control group (■). On the 33rd day after inoculation, all of the mice were killed. The (a) mice and (b) tumor appearance were photographed, and (d) the tumor masses were weighed. *$P < 0.01$ versus the other groups.

As cells approach the G_1–S transition, RB is phosphorylated and dissociates from E2F1. In turn, E2F1 is activated and bind to E2F1-responsive promoters, previously inhibited by RB-E2F-a complexes, and activated transcription. Cyclin A is the E2F/DP target gene, and E2F1 is an important upstream regulator of cyclin A.

The E2F family of transcription factors currently has eight members[28] including the activating E2Fs (E2F1-3a) and the repressing E2Fs (E2F3b-8). Overexpression of activator E2F is not only able to drive cells out of quiescence, but in some settings it also confers transforming potential to primary cells.[24]

In our study, western blotting showed that Ad-SSAT could decrease the protein level of E2F1. Using a luciferase reporter plasmid containing an E2F promoter, we showed that Ad-SSAT reduced the E2F promoter activity. Therefore, we presume that Ad-SSAT downregulates E2F1 expression by inhibition, which prevents E2F1 activation.

In summary, our data indicate that the upregulation of SSAT by Ad-SSAT can significantly suppress gastric cancer cell growth *in vitro* and have a significant *in vivo* growth-inhibitory effect. The inhibitory effects of Ad-SSAT on MGC803 and SGC7901 cells are mediated through downregulation of the cyclin A–E2F signaling pathway, which causes cell cycle arrest in S phase. Our results suggest that the upregulation of SSAT mediated by Ad-SSAT may be a potential therapeutic approach for gastric cancer.

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