

Lentivirus-mediated inhibition of USP39 suppresses the growth of gastric cancer cells via PARP activation

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Abstract. Gastric cancer (GC) is the second most common cause of cancer-associated mortality worldwide. Ubiquitin-specific peptidase 39 (USP39) has important roles in mRNA processing and has been reported to be involved in the growth of breast cancer cells. However, the roles of USP39 in GC have remained to be investigated, which was the aim of the present study. A lentivirus expressing short hairpin RNA targeting USP39 was constructed and transfected into MGC80-3 cells. Suppression of USP39 expression significantly decreased the proliferation and colony forming ability of MGC80-3 cells as indicated by an MTT and a clonogenic assay, respectively. In addition, flow cytometric cell cycle analysis revealed that depression of USP39 induced G2/M-phase arrest, while an intracellular signaling array showed that the cleavage of PARP at Asp214 was increased following USP39 knockdown. These results suggested that USP39 is involved in the proliferation of GCs and may be utilized as a molecular target for GC therapy.

Introduction

Gastric cancer (GC) is the second leading cause of cancer-associated mortality, despite its steady declining trend in most parts of the world. A total of 989,600 newly diagnosed cases of GC and 738,000 mortalities have been estimated for 2008, accounting for 8% of all newly diagnosed cancer and 10% of all cancer-associated mortalities (1). >70% of new diagnoses and mortalities occur in developing countries. Eastern Asia, Eastern Europe and South America have the highest incidence rates of GC, while rates are low in North America and most parts of Africa (1). As early GC is typically asymptomatic and features a small tumor size, it is usually detected at advanced stages (2,3). Therapeutic interventions to treat such late-stage

tumors are often restricted to non-curative gastrectomy, lymphadenectomy and post-operative chemoradiotherapy (4). Thus, the prognosis is poor and the five-year relative survival rate for GC is <30% in most countries (5). It is therefore of great clinical importance to identify novel biomarkers for early diagnosis and targeted treatment of GC.

Small ubiquitin-like modifiers (SUMOs) are highly conserved 11-kDa proteins that are detected in nearly all tissues of eukaryotic organisms, covalently attach and detach from target proteins to regulate their functions. SUMOylation, which is highly dynamic and reversible, is a type of post-translational modification and regulates numerous cellular functions and processes, including protein stability, protein localization, protein-protein interaction, cell cycle progression, DNA replication and repair, chromatin organization, transcription and RNA metabolism (6-8). Numerous diseases are associated with SUMO conjugation, including brain ischemia, heart failure, arthritis, degenerative diseases and cancer (9). DeSUMOylation enzymes are proteases that remove SUMOs from their substrate proteins and reverse the SUMOylation modification of target proteins, which counteracts ubiquitination (10,11). Ubiquitin-specific peptidase 39 (USP39), which is a 65-kDa SR-related protein of the U4/U6·U5 triple small nuclear ribonucleoprotein, is involved in the assembly of the mature spliceosome (12). USP39 is required to maintain the spindle checkpoint and sustain successful cytokinesis during the splicing of Aurora B (13). Mutation of USP39 in zebrafish has been shown to cause Rb1 mRNA splicing defects and pituitary lineage expansion (14). In addition, a previous study indicated that USP39 is overexpressed in human breast cancer tissues compared with that in normal breast tissues, and lentivirus-mediated suppression of USP39 inhibited the growth of breast cancer cells *in vitro* (15). Furthermore, USP39 was found to be a target protein of SUMO and mutation of its SUMOylation sites (K6, K16, K29, K51 and K73) was observed to promote the effects of USP39 on the proliferation of prostate cancer cells (16). However, the role of USP39 in GC has remained largely elusive.

In the present study, the expression of USP39 in the MGC80-3 GC cell line was knocked down using a lentivirus stably expressing small hairpin (sh)RNA targeting USP39 and the resulting effects on the proliferation, colony formation capacity and cell cycle were investigated. Knockdown of USP39 was found to markedly reduce the proliferation and colony formation capacity of MGC80-3-cells and to induce G2/M-phase arrest

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through regulating the cleavage of poly(adenosine diphosphate ribose) polymerase (PARP).

Materials and methods

Cell culture. MGC80-3, SGC-7901 and AGS human gastric cancer cell lines, and the 293T human embryonic kidney cell line were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). The MGC80-3 and SGC-7901 cell lines were cultured in 1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), and AGS were cultured in F12 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit-Haemek, Israel) and the 293T cell line was cultured in Dulbecco's modified Eagle's medium (Hyclone) containing 10% FBS. Cells were kept in a humidified atmosphere containing 5% CO₂ at 37°C.

Lentiviral vector construction and transfection. The construction of vectors was performed previously described (17) shRNA targeting the USP39 sequence and a scrambled control shRNA were cloned into the pFH-L lentiviral vector (Shanghai Hollybio, Shanghai, China). Briefly, DNA oligonucleotides were synthesized, annealed and inserted into the pFH-L vector by double digestion sites *Nhe*I and *Pac*I (Takara Biotechnology Co., Ltd., Dalian, China), and ligated with T4 DNA ligase (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. The ligation products were transformed in *E. coli* competent cells (Beijing Transgen Biotech Co., Ltd., Beijing, China), which were then cultured on coated plates and selected for monoclonal. Recombination of lentiviral expression vectors were confirmed by DNA sequencing. Their target sequences, synthesized by Genewiz, Inc. (South Plainfield, NJ, USA) were as follows: shUSP39, 5'-GATTTG GAAGAGGCGAGATAACTCGAGTTATCTCGCCTCTTCC AAATCTTTTT-3'; and shCon, 5'-GCGGAGGGTTTGGAA GAATATCTCGAGATATTCTTTCAAACCCTCCGCTTT TTT-3'.

For lentivirus production, 293T cells were transfected with 10 µg pFH-L lentiviral vectors along with 7.5 µg envelope plasmid pVSVG-I (Shanghai Hollybio) and 5 µg packaging plasmid pCMVΔR8.92 (Shanghai Hollybio) via Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h of incubation, the culture medium containing the lentivirus was collected.

To perform USP39 knockdown in MGC80-3 cells, 5x10⁴ cells were cultured in six-well plates and the USP39 shRNA-expressing lentivirus (shUSP39) or scrambled control shRNA-expressing lentivirus (shCon) was added with a multiplicity of infection of 60. After 96 h of transfection, cells were observed by fluorescence microscopy (#CKX41; Olympus, Tokyo, Japan). Positive cells were identified based on the expression of green fluorescence protein (GFP).

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using TRIzol reagent (Invitrogen). RT reaction was performed using 2 µg total RNA in a reaction mixture containing 2 µl oligo dT primers (50 µM), 4 µl 5X Moloney-Murine Leukemia Virus

(M-MLV) buffer, 1 µl dNTPs (10 mM), 0.5 µl RNasin, 0.5 µl M-MLV RT (RNase H-) and nuclease-free water in a total volume of 20 µl. The reaction mixture was incubated at 42°C for 1 h, 75°C for 15 min, then refrigerated on ice according to the M-MLV RT protocol. RT reagents was obtained from Promega Corporation (Madison, WI, USA).

PCR was performed using a CFX96 Touch Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. The primers used (synthesized by Genewiz, Inc.) were as follows: USP39 forward, 5'-GCCAGCAGAAGAAAAGAGC-3' and reverse, 5'-GCCATTGAACTTAGCCAGGA-3'; ACTB forward, 5'-GTGGACATCCGCAAAGAC-3' and reverse, 5'-AAAGGGTGTAACGCAACTA-3'. Forward and reverse primers were mixed and diluted to 2.5 µM. The PCR reaction mixture contained 0.8 µl primers, 5 µl cDNA (30 ng/µl), 10 µl 2X SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.) and 4.2 µl RNA-free water in a total volume of 20 µl. All reactions were performed in triplicate. Thermal cycling conditions comprised initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing extension at 60°C for 20 sec. Expression levels were normalized to the internal control ACTB. Relative quantification was performed using the 2^{-ΔΔC_q} method (18).

Western blot analysis. MGC80-3 cells were harvested after lentiviral transfection for five days. Cells were washed with phosphate-buffered saline (PBS; Sangon Biotech Co., Ltd., Shanghai, China) and lysed with ice-cold 2X sodium dodecyl sulfate (SDS) lysis buffer containing 100 mM Tris-HCl, pH 6.8, 10 mM ethylenediaminetetraacetic acid, 4% sodium dodecyl sulfate (SDS) and 10% glycine (all from Sangon Biotech Co., Ltd.), followed by 30 min of incubation on ice and centrifugation at 10,800 x g for 5 min at 4°C. The protein concentration was determined using the bicinchoninic acid protein assay (Beyotime Institute of Biotechnology Inc., Haimen, China). Protein extracts (30 µg/lane) were separated on a 10% SDS-polyacrylamide gel (Genscript, Nanjing, China) and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk in Tris-buffered saline containing Tween 20 (TBS-T; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature, the membranes were incubated with rabbit anti-USP39 antibody (cat. no. 23865-1-AP; 1:1,000 dilution; Proteintech Group, Inc., Chicago, IL, USA) or rabbit anti-glyceraldehyde-3-phosphate dehydrogenase antibody (cat. no. 10494-1-AP; 1:100,000 dilution; Proteintech Group, Inc.) at 4°C overnight and washed 3 times with TBS-T. Subsequently, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (cat. no. SC-2054; 1:5,000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and blots were developed with enhanced chemiluminescence reagent (RPN2132; GE Healthcare, Little Chalfont, UK) and exposed to X-ray film (Kodak, Rochester, NY, USA).

Cell proliferation assay. Cell proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After transfection with lentivirus for four days, MGC80-3 cells stably transfected with shCon or

shUSP39 (2,000 per well) were cultured in 96-well plates with five parallel wells per condition. On days 1, 2, 3, 4 and 5, 20 μ l MTT (5 mg/ml in PBS) was added, followed by incubation for 4 h. The reaction was terminated by addition of acidic isopropanol [10% SDS, 5% isopropanol (Sangon Biotech Co., Ltd.) and 0.01 mol/l HCl (Sangon Biotech Co., Ltd.)], and the absorbance was measured at a wavelength of 595 nm using an Epoch microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Colony formation assay. Following four days of lentiviral transfection, MGC80-3 cells were trypsinized, re-suspended and seeded into six-well plates at a concentration of 400 cells/well. Cells were cultured for seven days with replacement of the media every two days. Subsequently, the cells were washed with PBS twice and fixed with 4% paraformaldehyde (Sigma-Aldrich). Following two further washes with PBS, cells were stained with 700 μ l crystal violet (Beyotime Institute of Biotechnology) for 5 min and washed three times with PBS. The colonies were counted under an Olympus CH2 microscope (Olympus Corporation, Tokyo, Japan). Three independent assays were performed.

Cell cycle analysis. Cell cycle analysis was performed using flow cytometry. After lentiviral infection for four days, MGC80-3 cells (2×10^5 per dish) were seeded into 6-cm dishes and incubated for three days until a confluency of 70% was reached. Following two washes with cold PBS, cells were fixed in ice-cold 75% ethanol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) overnight at 4°C. Following washing in pre-cooled PBS, cells were stained with propidium iodide (Sigma-Aldrich) for 1 h at 37°C in the dark. Cell cycle distributions were determined using a Gallios flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). All experiments were performed in triplicate.

Intracellular signaling array. Following lentiviral transfection for five days, MGC80-3 cells were lysed in Cell Lysis Buffer (cat. no. 7018; Cell Signaling Technology, Inc., Danvers, MA, USA). Cell lysates were assayed using the PathScan Intracellular Signaling Array kit (cat. no. 7323; Cell Signaling Technology, Inc.) for simultaneous detection of phosphorylation or cleavage of 18 important and well-characterized signaling molecules according to the manufacturer's instructions. In brief, the lysate was diluted to 0.2 mg/ml in array diluent buffer (Cell Signaling Technology, Inc.), and 75 μ l lysate was added onto a nitrocellulose-coated glass slide pre-coated with capture antibodies. Following incubation of the slide overnight at 4°C, biotinylated detection antibody cocktail was added, following incubation for 1 h at room temperature. The slide was then washed 3 times with 1X array wash buffer (Cell Signaling Technology, Inc.) for 5 min at room temperature. Subsequently, HRP-linked streptavidin was added and the plate was incubated for 30 min at room temperature. Finally, chemiluminescent substrate was added and the signals were detected using X-ray film.

Statistical analysis. Values are expressed as the mean \pm standard deviation. Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla,

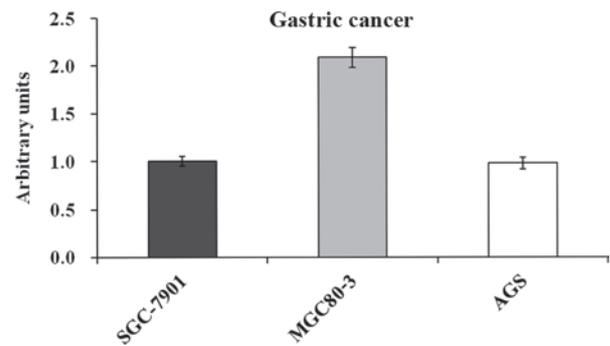


Figure 1. Expression of USP39 in three human gastric cancer cell lines, SGC-7901, MGC80-3 and AGS according to reverse-transcription quantitative polymerase chain reaction analysis. ACTB was used as the internal control. Values are expressed as the mean \pm standard deviation.

CA, USA) Statistical significance was determined using the two-tailed Student's *t*-test. $P < 0.05$ was considered to indicate a statistically significant difference between values.

Results

MGC80-3 cells express USP39 mRNA at high levels. Since USP39 levels had not previously been assessed in gastric cancers, the present study used RT-qPCR to determine USP39 expression in three human GC cell lines. As shown in Fig. 1, USP39 was expressed in the SGC-7901, MGC80-3 and AGS cell lines, with MGC80-3 cells expressing USP39 at markedly higher levels than the other two cell lines.

Knockdown of USP39 in MGC80-3 cells with the shRNA lentivirus system. To elucidate the functional role of USP39 in gastric cancer, sequences encoding for the expression of shUSP39 or shCon were cloned into the pFH-L lentiviral vector. The shUSP39 and shCon lentiviruses expressing GFP were generated and individually transfected into MGC80-3 cells. The transfection efficiency of the lentivirus was 95% after four days of transfection (Fig. 2A). RT-qPCR analysis revealed that transfection with shUSP39 reduced the mRNA levels of USP39 by 83% (Fig. 2B). In addition, western blot analysis revealed that in MGC80-3 cells transfected with the shUSP39 construct, the protein levels of USP39 were obviously reduced (Fig. 2C). These results suggested that the lentivirus stably expressing shRNA targeting USP39 was successfully constructed and transfected, and that it efficiently suppressed the expression of USP39.

USP39 knockdown inhibits GC-cell proliferation and colony formation. To investigate the effects of lentivirus-mediated downregulation of USP39 on the growth of GC cells, MGC80-3 cell proliferation was assessed using an MTT assay. As shown in Fig. 3A, USP39 knockdown completely inhibited the proliferation of MGC80-3 cells at days 2-5, while proliferation in the shCon group was not significantly different from that in the Con group ($P < 0.001$). On days four and five, cell growth in the USP30 knockdown group was reduced by 83.2 and 90.5%, respectively, compared with that in the control groups. Furthermore, a clonogenic assay showed that in the shUSP39 group, colony formation was completely inhibited, as

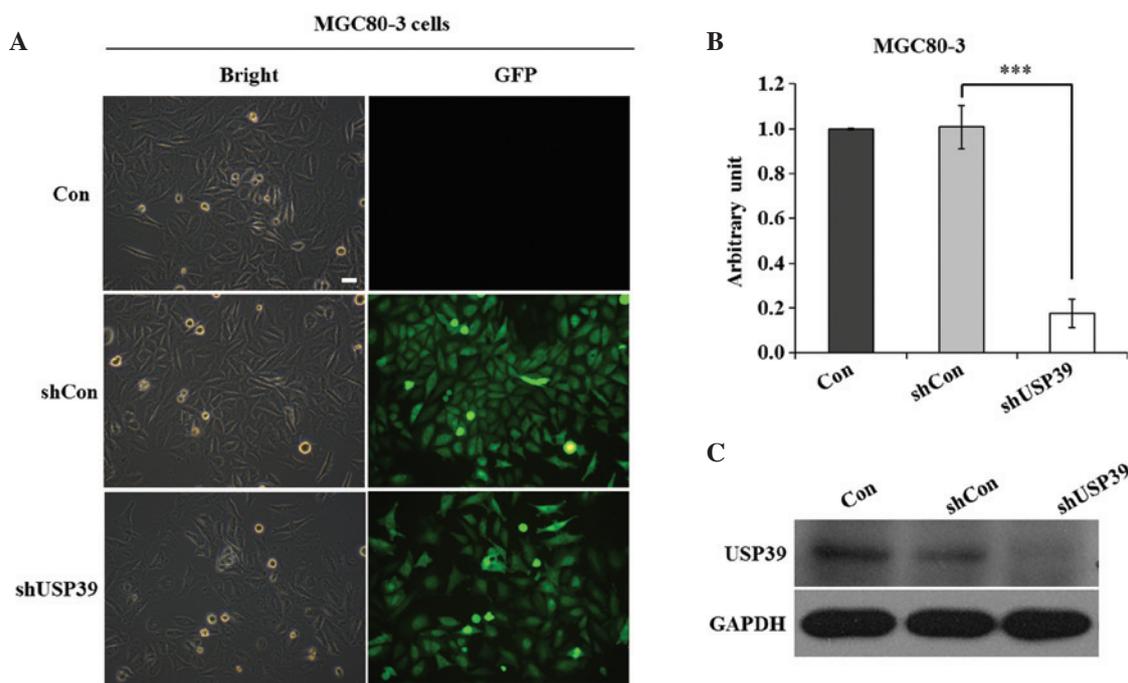


Figure 2. MGC80-3 cells stably expressing shUSP39 show significantly suppressed mRNA and protein expression of USP39. (A) Detection of lentiviral infection efficiency. Infection was observed to be efficient at 96 h and 95% of MGC80-3 cells presented to be positive for green fluorescence protein in the shCon and shUSP39 groups (scale bar, 10 μ m). (B) Reverse-transcription quantitative polymerase chain reaction analysis of USP39 mRNA levels verified that shUSP39 was successfully infected into MGC80-3 cells. Expression intensity of USP39 was normalized to ACTB. Values are expressed as the mean \pm standard deviation from three independent experiments. *** P <0.001. (C) Identification of knockdown efficiency in MGC80-3 cells using shUSP39 by western blot analysis. GAPDH was used as the loading control. Con, control; shCon, scrambled small hairpin RNA; shUSP39, small hairpin RNA targeting USP39; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

no colonies were formed over the assay period of seven days, while colony size in the shCon group was equal to that in the Con group (Fig. 3B). In addition, the number of colonies in the shCon and Con groups was similar, while no colonies were formed in the shUSP39 group (Fig. 3C). These findings indicated that the knockdown of USP39 completely inhibited the cell proliferative ability and colony formation capacity of GC cells.

Suppression of USP39 causes cell cycle arrest in G2/M phase. Based on the observed repression of MGC80-3-cell proliferation and colony formation by shUSP39, flow cytometric analysis was performed to observe the effects of USP39 on cell cycle progression (Fig. 4A). The results demonstrated that after transfection with shUSP39 lentivirus, the G0/G1-phase population accounted for 35.0%, which was obviously decreased compared to that in the shCon group (56.1%; P <0.001), while the G2/M-phase population in the shUSP39 group was increased (40.8 vs. 19.1% in shCon group; P <0.001) (Fig. 4B). This result suggested that USP39 depletion causes G2/M-phase arrest in GC cells.

USP39 silencing induces the cleavage of PARP. To reveal the mechanisms governing the inhibitory effects of shUSP39 on cell growth, the PathScan Intracellular Signaling Array kit was used to detect 18 important and well-characterized signaling molecules in the shUSP39- or shCon-transfected MGC80-3 cells. The results showed that the cleavage of PARP at Asp214 was markedly enhanced in the shUSP39-transfected MGC80-3

cells, indicating that the cleavage of PARP may be involved in shUSP39-mediated growth suppression in GC cells.

Discussion

Understanding the molecular aberrations behind the initiation and progression of GC is important in finding novel molecular markers for early diagnosis, targeted treatment and prognosis evaluation. Overexpression of USP39 has been reported in human breast cancers, and inhibition of USP39 repressed the growth of breast cancer cells (15). However, to date, the functional role of USP39 in GC has remained elusive.

To the best of our knowledge, the present study was the first to shed light on the functional role of USP39 in GC. A lentiviral shRNA system was used to effectively inhibit the expression of USP39 at the mRNA and protein level. RT-qPCR and western blot analysis showed efficient silencing of USP39. An MTT assay and a colony formation assay were then used to identify the effects of USP39 knockdown on GC-cell proliferation. USP39 deletion was shown to completely inhibit the proliferation and colony formation ability of MGC80-3 cells. The present study also revealed that knockdown of USP39 caused G2/M-phase arrest in MGC80-3 cells. Furthermore, the PathScan Intracellular Signaling Array indicated that inhibition of USP39 increased the cleavage of PARP.

USP39, containing a central zinc finger and two ubiquitin C-terminal hydrolase domains, belongs to the ubiquitin-specific protease family (14). Previous studies have shown that USP39 is overexpressed in breast cancer

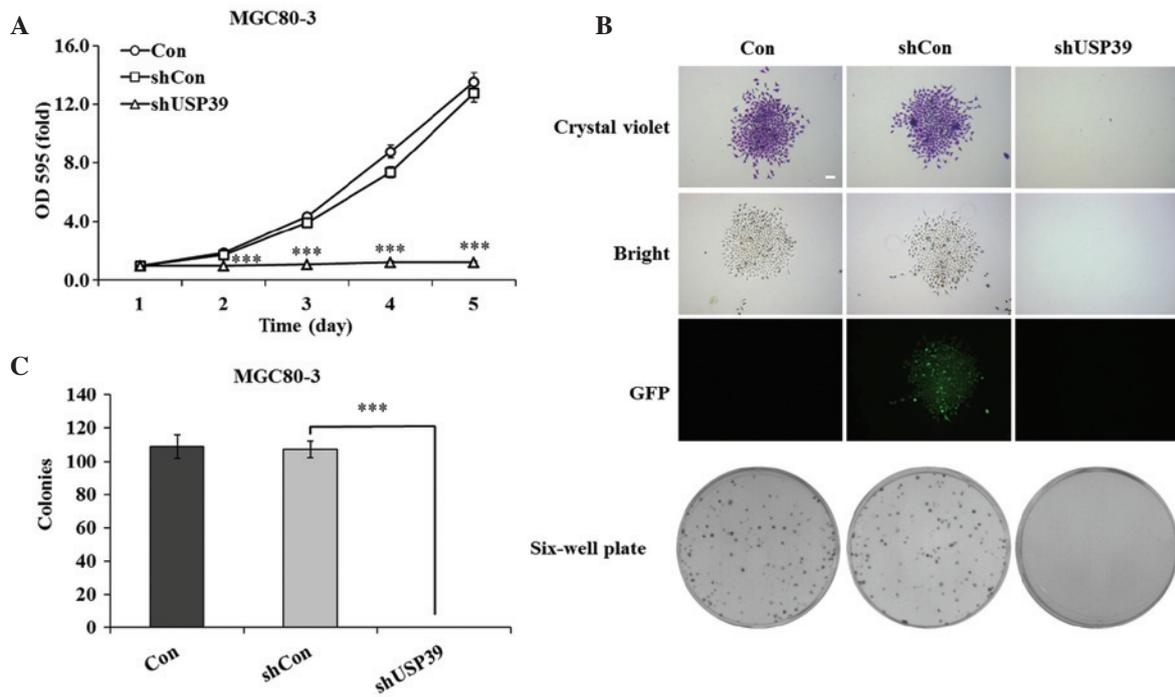


Figure 3. USP39 depletion inhibits MGC80-3 cell proliferation and colony formation. (A) The proliferation of USP39-knockdown cells was significantly decreased on days 4 and 5 compared with the shCon and Con cells according to an MTT assay. (B) USP39 silencing markedly suppressed colony formation of gastric cancer cells (scale bar, 25 μ m). (C) USP39 depletion significantly lowered the numbers of formed colonies. Values are expressed as the mean \pm standard deviation. *** P <0.001. GFP, green fluorescence protein; Con, control; shCon, scrambled small hairpin RNA; shUSP39, small hairpin RNA targeting USP39; OD, optical density.

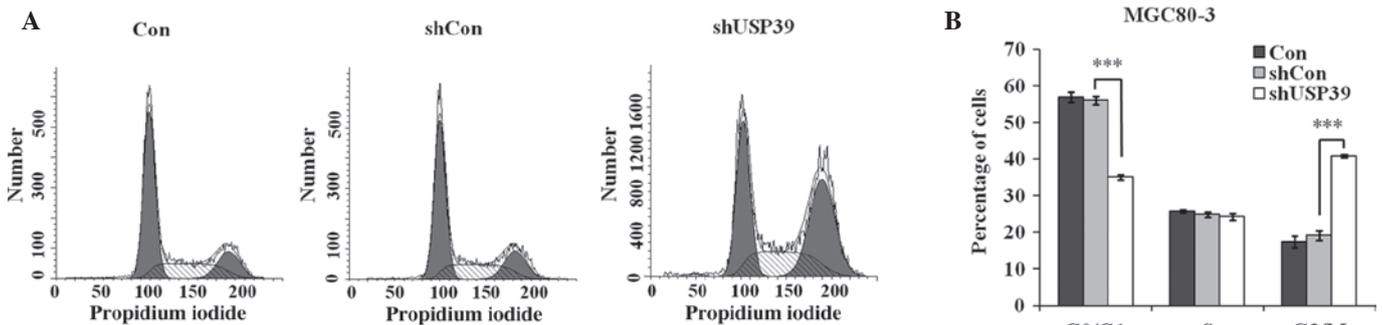


Figure 4. Knockdown of USP39 induces cell cycle arrest of MGC80-3 cells in G2/M-phase. (A) Cell cycle progression in the Con, shCon and shUSP39 groups was assessed by flow cytometry. (B) Quantification of the percentage of cells in cell cycle phases G0/G1, S and G2/M. After transfection with shUSP39-expressing lentivirus, the G0/G1-phase population was obviously decreased and the G2/M-phase population was increased. Values are expressed as the mean \pm standard deviation of three independent experiments. *** P <0.001. Con, control; shCon, scrambled small hairpin RNA; shUSP39, small hairpin RNA targeting USP39.

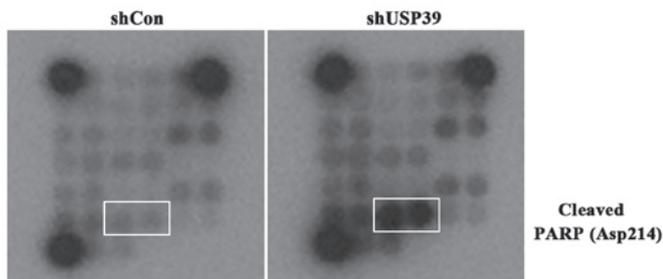


Figure 5. Intracellular signaling array of USP39-silenced and negative control groups. The cleavage of PARP at Asp214 was markedly enhanced in the shUSP39-transfected MGC80-3 cells. Con, control; shCon, scrambled small hairpin RNA; shUSP39, small hairpin RNA targeting USP39; PARP, poly(adenosine diphosphate ribose) polymerase.

tissues and that knockdown of USP39 obviously inhibits the proliferative and colony formation ability of MCF-7 breast cancer cells. In addition, USP39 knockdown was revealed to induce G0/G1-phase arrest and apoptosis of breast cancer cells (15). However, the present study showed that shUSP39 effectively inhibited GC-cell growth and colony formation, possibly by causing cell cycle arrest in G2/M phase. Recently, Wen *et al* (16) indicated that the overexpression of USP39 increased the proliferation of the androgen-independent PC3 and androgen-dependent LNCaP prostate cancer cells. Of note, mutation of the SUMOylation sites of USP39 was demonstrated to further strengthen its ability to promote the proliferation of prostate cancer cells. These results suggested

that SUMO modifications have a significant role in the function of USP39.

To reveal the underlying mechanisms of the effects of shUSP39 on GC-cell growth, the present study screened for intracellular signaling target proteins by using a high-throughput proteomics method. The cleavage of PARP (Asp214) was found to be obviously increased in shUSP39-transfected MGC80-3 cells. PARP1, also known as PARP, is involved in DNA repair. As USP39 is required to maintain the spindle checkpoint and sustain successful cytokinesis during splicing (13,14), it is likely that loss of USP39 led to a halt or dysregulation of mitosis, which explains for the observed G2/M-phase arrest. PARP may have been activated due to DNA damage or faulty splicing in the absence of USP39. Indeed, PARP, which is involved in DNA repair, is cleaved during apoptosis. The cleavage of PARP often occurs between Asp-214 and Gly-215 and has been demonstrated to be an early marker of apoptosis (19). PARP-1 762Ala/Ala has been indicated to be a risk factor for GC in a Han Chinese population (20), and the PARP1 rs1136410 genotype has been correlated with the risk of lymph node metastasis and tumor invasion in GC (21). It was previously reported that PARP expression in yeast was increased during the G2/M phase of the cell cycle (22). Furthermore, G2/M arrest was enhanced by PARP inhibitors in mammalian cells (23,24). However, the underlying mechanism of PARP activity in the regulation of G2/M phase arrest remains unclear and requires clarification in future studies. Taken together, the results of the present study indicated that USP39 knockdown caused a de-regulation of mitosis, resulting in G2/M-phase arrest and cleavage of PARP (Asp214) for DNA damage repair, leading to total inhibition of GC cell proliferation. However, it remains to be clarified whether inhibition of USP39 affects normal cells in patients, leading to considerable side effects (13,14).

In conclusion, the present study was the first to reveal that shRNA-mediated knockdown of USP39 inhibited the growth and colony formation ability of GC cells. Furthermore, suppression of USP39 induced G2/M-phase arrest and increased the cleavage of PARP (Asp214). The present study suggested that USP39 is crucial for the proliferation of GC cells. Due to its upregulated expression in certain cancer types and with overproliferation being a hallmark of cancer, USP39 may be targeted for the treatment of cancer. Future studies assessing USP39 in normal and cancer cells will reveal whether USP39 is a feasible and cancer-specific molecular target.

Acknowledgements

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