Downregulation of SENP1 inhibits cell proliferation, migration and promotes apoptosis in human glioma cells

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Abstract. Small ubiquitin-related modifier protein (SUMO) is an evolutionarily conserved protein in a broad range of eukaryotic organisms. De-SUMOylation, the reverse reaction of SUMOylation, is regulated by a family of SUMO-specific proteases (SENPs). SENP1 is a member of the de-SUMOylation protease family involved in the de-SUMOylation of a variety of SUMOylated proteins. The present study demonstrates that small hairpin RNA (shRNA)-mediated downregulation of SENP1 inhibits cell proliferation and migration, and promotes apoptosis in human glioma cells. Firstly, LN-299 cells were transfected with a plasmid expressing SENP1 shRNA (pGenesil-1-SENP1). The messenger RNA and protein expression of SENP1 was detected by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. Cell proliferation *in vitro* was assessed using a methyl thiazolyl tetrazolium assay. Flow cytometry (FCM) was used to detect the apoptosis of LN-299 cells. The effect of the downregulation of SENP1 on cell migration was detected by a Transwell migration system. The present results showed that, compared with the control shRNA group, the expression of SENP1 was significantly reduced in the SENP1 shRNA group. The proliferation was markedly inhibited in the SENP1 shRNA group. FCM findings revealed that apoptosis increased significantly in the SENP1 shRNA group. In addition, it was found that downregulation of SENP1 evidently suppressed tumor cell migration. Downregulation of SENP1 expression inhibited the proliferation and migration and promoted apoptosis in LN-299 cells. These results indirectly demonstrate that SENP1 is likely to play a critical role in human glioma cells.

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Introduction

Conjugation of small ubiquitin-related modifier protein (SUMO) has been reported in organisms, including *Arabidopsis thaliana* and mammals, indicating that SUMO is an evolutionarily conserved protein that may have unique functions in cellular metabolism (1). By conjugating to protein substrates, SUMO regulates the localization and activity of target proteins (1-5). SUMO modification is involved in numerous metabolic processes in cells and plays an important role in the balance and interaction between proteins, transcriptional activity and cellular localization (4,6-8). De-SUMOylation, the reverse reaction of SUMOylation, is regulated by the SENP family (9). Once the balance between SUMOylation and de-SUMOylation is broken, there will be an overexpression of SUMO or SENPs in cells, which may lead to tumor development.

There are 6 SENPs in mammals (10). SENP1, a nuclear protease, promotes de-SUMOylation processing of a variety of proteins that have been SUMOylated. The majority of cases of prostate cancer demonstrate an increased expression of SENP1 (11-13). Subsequent to the comparison of SENP1 levels between normal prostate and prostate cancer tissues, a previous study found that increased expression of SENP1 was observed in 60% of patients with prostate cancer (13). Androgens and interleukin-6 easily increased SENP1 expression in prostate cancer (14). SENP1 induction mediates cell proliferation by increasing androgen receptor-dependent transcription, c-Jun-dependent transcription and cyclin D1 levels (14-16). However, the mechanism of SENP1 in human glioma cells remains unclear. Since SUMO and proteins modified by SUMO may be important in the occurrence of malignant glioma (17), it is essential to define the role of SENP1 in human glioma cells, which may aid in the identification of potential therapeutic targets for malignant glioma. Therefore, the present study aimed to define the role of SENP1 in human glioma cells.

Materials and methods

Main reagents. Human glioma LN-299 cells were purchased from the American Type Culture Collection (catalog no., CRL-2611; Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) with high glucose, fetal bovine serum (FBS) and phosphate buffer solution (PBS) were obtained

from HyClone (GE Healthcare, Logan, UT, USA). Cell culture dishes were purchased from Corning Life Sciences (Corning, NY, USA). TRIzol reagent and First Strand cDNA Synthesis kit were purchased from Tiangen Biochemical Technology Co., Ltd. (Beijing, China). Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection kit was purchased from Nanjing KeyGen Biotech Co., Ltd. (catalog no., KGA106; Nanjing, Jiangsu, China). Rabbit anti-SENP1 monoclonal (catalog no., ab108981), rabbit anti-cyclin D1 monoclonal (catalog no., ab134175), rabbit anti-c-Jun monoclonal (catalog no., ab32137) and rabbit anti-β-actin polyclonal (catalog no., ab8227) antibodies were from Abcam (Cambridge, MA, USA).

shRNA design and expression plasmid vector construction. Two shRNAs targeting the SENP1 gene were synthesized: SENP1 shRNA-1, 5'-GCACCTCATCAGCCAAATAGC-3'; and SENP1 shRNA-2, 5'-GCATTCCGCTTGACCATT ACA-3'. shRNA targeting scrambled sequence (general sequence, 5'-TTCTCCGAACGTGTCACGT-3') was designed and acted as the negative control group. The DNA sequence targeting the SENP1 gene was cloned into the pGenesil-1 vector (catalog no., VRG0358; Wuhan Genesil Biotechnology Co., Ltd., Wuhan, Hubei, China), which expresses shRNA and enhanced green fluorescent protein (EGFP) in mammalian cells. The recombinant plasmids pGenesil/SENP1 and pGenesil/NC were constructed and verified by Wuhan Genesil Biotechnology Co., Ltd.

Cell culture and transfection. Glioma cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS. All cells were cultured in a humidified incubator, containing 5% CO_2 at 37˚C. LN-299 cells were replated at a density of $5x10^6$ cells/well in 6-well plates. When the cell density reached 40-50%, cells were transfected with SENP1 shRNA using Lipofectamine 2000 (Invitrogen™; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Messenger RNA (mRNA) transcript expression was quantified RT-qPCR and normalized against the expression of β-actin. Using 1 ml TRIzol, cell lysis was performed at room temperature for 5 min, followed by treatment with 0.2 ml chloroform (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at room temperature. The aforementioned mixture was centrifuged at 12,000 x g for 15 min at 4˚C, and the supernatant was then mixed with 0.4 ml isopropyl alcohol and placed for 10 min at room temperature. The centrifugal sedimentation was obtained by centrifugation at 12,000 x g for 10 min at 4˚C, was washed with diethylpyrocarbonate containing 75% ethanol and placed at room temperature to dry. The quality of the RNA was confirmed using an absorbance cut-off of A260/A280>1.8 (ND-2000; NanoDrop™, Thermo Fisher Scientific, Inc.). RT-qPCR was performed using an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following cycling conditions: 5˚C for 1 min, followed by 40 cycles of 95˚C for 10 sec, 55˚C for 20 sec and 72˚C for 5 sec. The following primers were used: SENP1 forward, 5'-CTACAAGAAGCCCAGCCTATCGTC-3' and reverse, 5'-GTCACCTGAGCCAAGGAAACTG-3'; and β-actin forward, 5'-CTTTCTACAATGAGCTGCGTG-3' and reverse, 5'-TCATGAGGTAGTCTGTCAGG-3'. Subsequent to confirmation of the quality of RNA, 2 μg RNA was reverse transcribed into cDNA.

Western blot analysis. Total protein was extracted from cell lines using the ReadyPrep Protein Extraction kit (catalog no., 163-2090; Bio-Rad Laboratories, Hercules, CA, USA). The protein concentration was determined using a Pierce BCA Protein Assay Kit (catalog no., 23227; Thermo Fisher Scientific, Inc.). Protein lysates were loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Separated protein bands were electro-transferred onto polyvinylidene fluoride (PVDF) membranes (catalog no., IPVH00010; EMD Millipore, Billerica, MA, USA). The PVDF membranes were then blocked in Tris-buffered saline (TBS; Neuromics, Inc., Minneapolis, MN, USA) containing 10 mM Tris-HCl (pH 7.5) and 150 mM NaCl, and 5% non-fat dry milk at room temperature for 1 h. The membranes were then incubated at 4˚C overnight with the rabbit anti-SENP1 monoclonal (catalog no., ab108981; dilution, 1:1,000; Abcam), rabbit anti-c-Jun monoclonal (catalog no., ab32137; dilution, 1:1,000; Abcam), rabbit anti-cyclin D1 monoclonal (catalog no., ab314175; dilution, 1:1,000; Abcam) and rabbit anti-β-actin polyclonal (catalog no., ab8227; dilution, 1:1,000; Abcam) antibodies. Subsequent to incubation with the primary antibody, the membranes were washed in TBS with 0.05% Tween-20 (Sigma-Aldrich), and the secondary goat anti-rabbit immunoglobulin G heavy and light chain horseradish peroxidase-conjugated antibody (catalog no., ab6721; dilution, 1:2,000; Abcam) was added. The membrane was then incubated at 37˚C for 2 h. Pierce ECL Plus Western Blotting Substrate (catalog no., 32132; Thermo Fisher Scientific, Inc.) was used to visualize the immunoreactive bands and Image J 1.42q software (National Institutes of Health, Bethesda, MA, USA) was used for quantification. Relative protein level was normalized against the β-actin concentration. Three separate experiments were performed in duplicate for each treatment.

Cell proliferation assay. Cell viability was assessed using a methyl thiazolyl tetrazolium (MTT) assay (Thiazolyl Blue Tetrazolium Bromide; catalog no., M2128; Sigma-Aldrich). Cells were cultured in 24-well plates at a concentration of $5x10^6$ cells per well and allowed to adhere. Subsequent to treatment at various time intervals (24, 48, 72 and 96 h), 100 μl MTT (0.5 mg/ml) was added to the cells and the mixture was incubated for 4 h at 37˚C. Subsequently, the supernatant was removed, and dimethyl sulfoxide (catalog no., D2650; Sigma-Aldrich) was used to dissolve the resultant formazan crystals. The absorbance value was read at 570 nm using a microplate reader (Automated Microplate Reader EL309; Bio-Tek Instruments, Inc., Winooski, VT, USA). Six wells were measured for each group, and the experiment was repeated three times.

Flow cytometry analysis of cell apoptosis. To detect cell apoptosis, the proliferating phase LN-299 was trypsinized, washed with cold PBS and resuspended in binding buffer using the Annexin V-FITC/PI Apoptosis Detection kit (catalog no., KGA106; Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's protocol. Annexin V-FITC

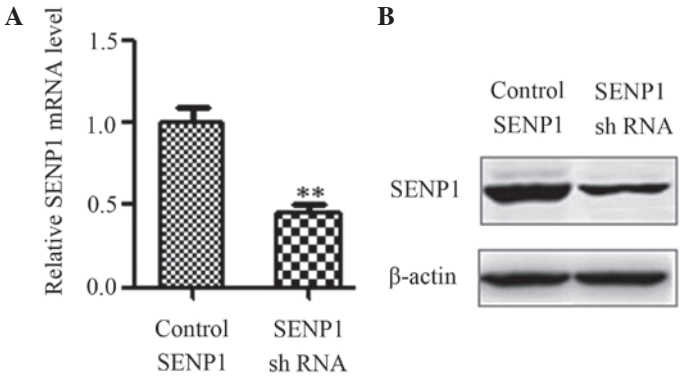

Figure 1. Relative mRNA and protein expression of SENP1 in human glioma LN-299 cells. The mRNA and protein expression of SENP1 was detected by (A) reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis, respectively. **P<0.01. mRNA, messenger RNA; SENP1, small ubiquitin-related modifier protein-specific protease 1; shRNA, short hairpin RNA; Control shRNA, pGenesil-1 scramble control group; SENP1 shRNA, pGenesil-1-SENP1-transfected group.

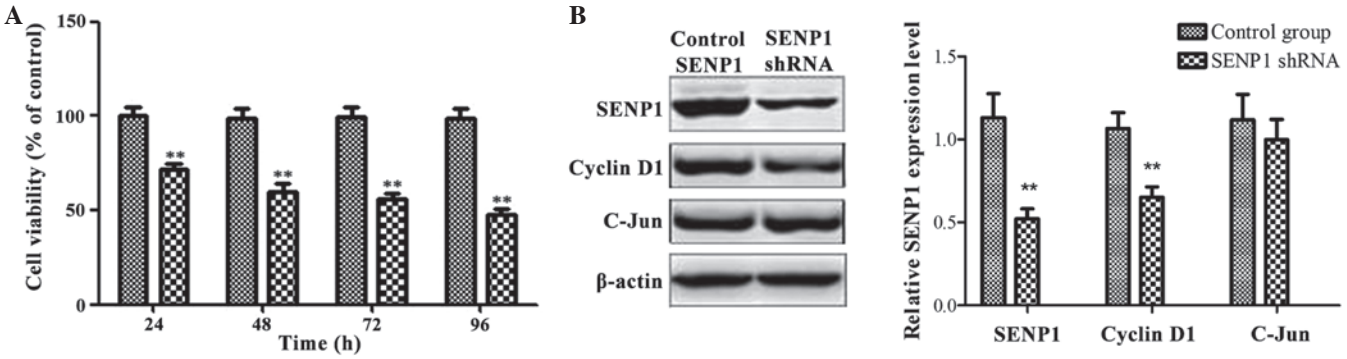

Figure 2. Downregulation of SENP1 inhibited cell proliferation in human glioma LN-299 cells. (A) Methyl thiazolyl tetrazolium assays showed that the proliferation of LN-299 pGenesil-1-SENP1-transfected cells was inhibited in a time-dependent manner. (B) Downregulation of SENP1 inhibited the expression of cyclin D1, suggesting that downregulation of SENP1 may inhibit tumor cell proliferation and viability by mediating the expression of cyclin D1. **P<0.01. SENP1, small ubiquitin-related modifier protein-specific protease 1; shRNA, short hairpin RNA; Control shRNA, pGenesil-1 scramble control group; SENP1 shRNA, pGenesil-1-SENP1-transfected group.

and PI were added to the fixed cells for 20 min in darkness at room temperature. Annexin V binding buffer was then added to the mixture prior to the fluorescence being measured on FACSort flow cytometer (BD Biosciences, San Jose, CA, USA). Cell apoptosis was analyzed using the Cell Quest 3.0 software (BD Biosciences).

Transwell migration assay. Cell culture inserts and Transwell chamber (catalog no., 3464; Corning Life Sciences, Tewksbury, MA, USA) were pre-warmed up at 37˚C. Cells in the logarithmic phase of growth were rinsed with PBS and adjusted to an appropriate concentration. Resuspended cells were cultured in the upper chamber of the Transwell inserts. The lower chambers contained ~600 μl culture medium containing 10% FBS. Following 24 h of incubation, transmigrated cells were fixed with 800 μl methyl alcohol and stained with Giemsa (Sigma-Aldrich) at room temperature for 30 min. The number of migratory cells were counted by capturing images of the membrane under a microscope (BX51-P; Olympus Corporation, Tokyo, Japan).

Statistical analysis. SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) was used for the data analysis. All experiments were repeated three times and data are presented as the mean ± standard deviation (SD). The data were compared between two groups using the two-tailed Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

SENP1 expression was downregulated by SENP1 shRNA. To knockdown SENP1, SENP1 shRNA and control shRNA were transfected into glioma cells. Subsequent to 48 h of culturing, RNA was extracted, and reverse transcription reactions were performed to illustrate various mRNA expression levels of SENP1. It was found that SENP1 shRNA effectively downregulated the mRNA levels of SENP1 (Fig. 1A; 0.45±0.04 vs. 1.00±0.14; P=0.02). Accordingly, the downregulation of the SENP1 protein was detectable by western blot analysis (Fig. 1B).

Downregulation of SENP1 inhibited cell proliferation and viability. The effect of the downregulation of SENP1 on the proliferation and viability of glioma cells was detected. The viable cells were counted using a cell counting chamber every 24 h for 4 days subsequent to transfection. The viable cells in the control shRNA group were defined as 1 x 100%, and data were expressed as the percentage of growth inhibition as follows:

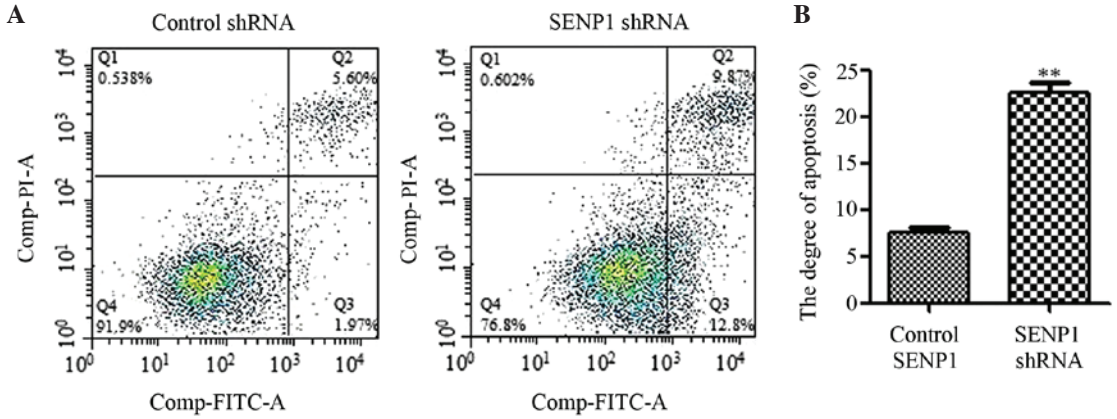

Figure 3. Downregulation of SENP1 induced apoptosis in human glioma cells. (A) Flow cytometry was used to detect the apoptosis of LN-299 cells. (B) Downregulation of SENP1 increased the apoptosis rate of human glioma cells. **P<0.01. SENP1, small ubiquitin-related modifier protein-specific protease 1; shRNA, short hairpin RNA; FITC, fluorescein isothiocyanate; PI, propidium iodide; Control shRNA, pGenesil-1 scramble control group; SENP1 shRNA, pGenesil-1-SENP1 transfected group.

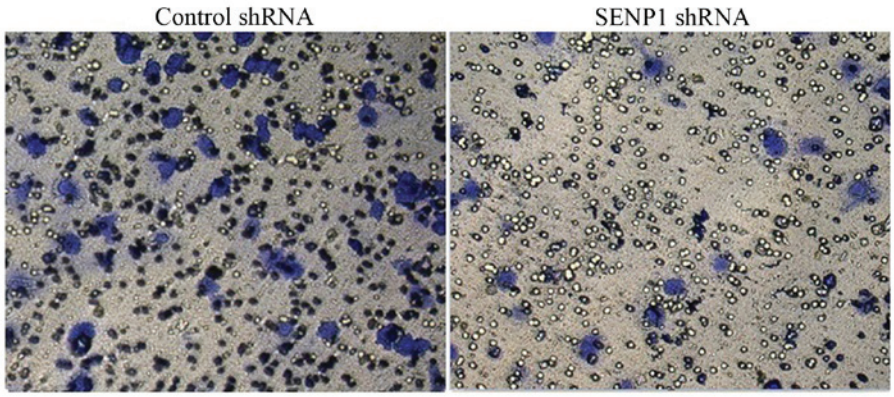

Figure 4. The effect of SENP1 downregulation on cell migration was detected by a Transwell migration assay, and it was found that downregulation of SENP1 suppressed cell migration. SENP1, small ubiquitin-related modifier protein-specific protease 1; shRNA, short hairpin RNA; Control shRNA, pGenesil-1 scramble control group; SENP1 shRNA, pGenesil-1-SENP1 transfected group.

Relative cell viability = A595 (SENP1 shRNA group) / A595 (control shRNA group) x 100%. Following knockdown of SENP1, the number of viable human glioma cells decreased in a time-dependent manner (Fig. 2A) (SENP1 shRNA group vs. control group: 24 h, 71.01±3.43 vs. 100±3.95%, P=0.0024; 48 h, 58.47±4.69 vs. 100±2.66%, P=0.0002; 72 h, 54.99±2.50 vs. 100±3.25%, P<0.0001; 96 h 46.21±2.81 vs. 100±2.59%, P<0.0001). Compared to the control group, the viable cells in the SENP1 shRNA group decreased to ~50% at 96 h subsequent to transfection (SENP1 shRNA group vs. control group: 100±2.59 vs. 46.21±2,81%, P<0.0001). These results indicated that downregulation of SENP1 may effectively kill tumor cells. To define the present conclusion, levels of cyclin D1 protein was determined by semi-quantified western blot analysis. As shown in Fig. 2B, SENP1 knockdown inhibited the expression of cyclin D1 to a certain degree, suggesting that SENP1 knockdown may inhibit tumor cell proliferation and viability by mediating the expression of cyclin D1.

Downregulation of SENP1 induced apoptosis. To explore the role of SENP1 in glioma cells, Annexin V-FITC/PI Apoptosis Detection kit (catalog no., KGA106; Nanjing KeyGen Biotech Co., Ltd.) was used for apoptosis analysis. SENP1 knockdown was found to significantly induce apoptosis in human glioma cells following 72 h of transfection (SENP1 shRNA group vs. control group, 22.46±0.91 vs. 7.43±0.72%; P<0.0001), in accordance with the inhibitory effect on cell proliferation and viability (Fig. 3).

Downregulation of SENP1 inhibited cell migration. Migration is one of the features of tumor cells. The effect of the downregulation of SENP1 on cell migration was investigated using a Transwell migration system. As the representative images show in Fig. 4, SENP1 knockdown was found to evidently suppress tumor cell migration (SENP1 shRNA group vs. control group, 37.93±8.29 vs. 100±5.78%; P<0.0001).

Discussion

Recently, SENP1 has been less frequently reported in studies. It has previously been reported that SENP1 was overexpressed in prostate cancer tissue samples from patients, and the overexpression of SENP1 contributes to the progression of prostate cancer (11). High expression of SENP1 is also associated with the occurrence and development of gastric cancer (18).

However, the effect of SENP1 on human glioma cells remains unclear.

In the present study, SENP1 was found to perform a vital function in human glioma cells. The results in this study indicate that SENP1 acts as a positive regulator in human glioma cell viability, migration and proliferation (Fig. 2A). This is consistent with the results of previous study (11), in which SENP1 was reported to affect the tumorigenesis of prostate cancer cells *in vivo* and *in vitro*. Decreased levels of the cyclin D1 protein were also detected in glioma cells to a certain degree following downregulation of SENP1, which suggested that SENP1 knockdown may suppress cell proliferation and viability by mediating the cell cycle. In addition, the present study showed that SENP1 knockdown significantly induced apoptosis in human glioma cells (Fig. 3) and suppressed tumor cell migration (Fig. 4), which was similar to the effects of SENP1 on prostate cancer and hepatocellular carcinoma cells as reported by previous studies (11,19). Two reasons may exist for cell migration suppression. One reason is that the migration-associated genes expression may be inhibited by the downregulation of SENP1 expression, and the other reason is that migrated cells became decreased in number due to the decrease in viable cells and increase in apoptotic cells. These results indirectly demonstrate that SENP1 is likely to play a critical role in human glioma cells, as shown by the results from additional studies (19,20). However, the definite mechanism of SENP1 in cell proliferation and apoptosis requires investigation in additional studies.

In conclusion, the present experimental results show the anti-tumor effects of SENP1 on the proliferation and apoptosis of human glioma LN-299 cells. SENP1 may be a potential therapeutic target for the inhibition of growth and progression of glioma cells. The present study may provide insight into the tumorigenesis and development of glioma cells and provide novel strategies and targets for glioma treatment. However, additional studies are required to clarify the associated molecular mechanisms and signal transduction of SENP1.

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