Inhibitory effects of plasmid small interfering RNA targeting signal transducer and activator of transcription-3 in C6 glioma cells

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Abstract. The current study investigated the effect of plasmid small interfering RNA (psiRNA)-mediated silencing of signal transducer and activator of transcription-3 (STAT3) on the invasion, apoptosis, and expression levels of cyclin D1, caspase-3 and B-cell lymphoma-2 (Bcl-2) in C6 glioma cells. Cell invasion was determined using a Transwell assay, while the apoptosis rate and cell cycle distribution of cells were assessed using Annexin V-FITC/PI double staining. The expression levels of cyclin D1, caspase-3 and Bcl-2 proteins were measured by western blotting. Transfection with psiRNA-STAT3 was observed to significantly decrease the number of transmembrane cells compared with the control groups ($P<0.05$). In addition, the proportion of cells at G0/G1 phase was increased in the psiRNA-STAT3 group compared with the controls. Western blotting indicated that psiRNA-STAT3 decreased the expression of cyclin D1, caspase-3 and Bcl-2 proteins. Taken together, psiRNA-STAT3 inhibited the migration and invasive abilities, and induced the apoptosis of C6 glioma cells, possibly through regulation of the expression of cyclin D1, caspase-3 and Bcl-2 proteins.

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Key words: plasmid small interfering RNA, signal transducer and activator of transcription-3, glioma cells, apoptosis, protein expression, cell cycle

Introduction

Gliomas are the most common malignant intracranial tumors of the human central nervous system (1,2), and their occurrence and development involve multi-step and multi-factorial processes. Traditional treatments, such as surgery, radiotherapy and chemotherapy, have low cure rates and high tumor recurrence rates, and are associated with poor prognosis (3). Various newly developed therapies, including genetic immunotherapy, microsurgery (4), new radiotherapy and chemotherapy strategies (5), anti-invasion therapy (6) and anti-angiogenesis therapy (7), have recently led to great progress in the treatment of gliomas and other malignant tumors (8,9). However, due to the characteristics of infiltrative growth (10), increased drug tolerance (11) and high invasiveness (12) of gliomas, these treatments have not achieved satisfactory results in all clinical trials (13), and patient prognosis has not been significantly improved.

Current novel antitumor treatment strategies aim to simultaneously target different aspects of the tumorigenesis mechanism to achieve better efficacy. Specific targeting of signal transducer and activator of transcription-3 (STAT3) signaling transduction has potential value for the treatment of malignant glioma (14).

In our previous study, it was demonstrated that transfection with STAT3-specific silencing RNA, namely plasmid small interfering RNA (psiRNA)-STAT3, induced apoptosis of glioma cells through the endogenous caspase pathway mediated by the mitochondrial membrane potential, thus inhibiting the growth of tumor cells (15). The present study aimed to further investigate the underlying mechanisms of the effects of psiRNA-STAT3 on C6 glioma cells in order to provide a theoretical basis for clinical research into glioma treatment.

Materials and methods

Cells and reagents. Rat C6 glioma cells were purchased from the Shanghai Institute of Cell Biology (Chinese Academy of Sciences, Shanghai, China). Opti-minimum essential medium

(MEM; Gibco) and Lipofectamine® 2000 reagent (Invitrogen; cat. no. 11668-019) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). psiRNA-STAT3 and scramble psiRNA were constructed at the Department of Pathophysiology of the Institute of Basic Medicine of Jilin University (Changchun, China). The si-STAT3 sequence was GCAGCAGCTGAACAACAT (spanning nucleotides 2,144-2,162; GenBank accession no. NM003150). Transwell chambers were obtained from Corning, Inc. (Corning, NY, USA). Pounceau S staining solution, Triton X-100, 5X sodium dodecyl sulfate (SDS) loading buffer and the Annexin V-FITC cell apoptosis detection kit (cat. no. GK3603-50T) were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). Ribonuclease (cat. no. ST577), BeyoECL Plus (cat. no. P0018S) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit anti-cyclin D1 (cat. no. BA0770), rabbit anti-B-cell lymphoma-2 (Bcl-2; cat. no. A00040-1), rabbit anti-caspase-3 (cat. no. BA2142), horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. BA1055) and anti-β-actin (cat. no. BM0626) antibodies, and propidium iodide were obtained from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Radioimmunoprecipitation assay (RIPA) buffer was purchased from Beyotime Institute of Biotechnology.

Instruments. The ECLIPSE 80i inverted microscope was obtained from Nikon Corporation (Tokyo, Japan), and the Guava easyCyte 6HT-2L flow cytometer powered by GuavaSoft 1.0 was from Merck KGaA (Darmstadt, Germany). The WH-600-LCD electrophoresis apparatus was purchased from Shanghai Qite Analytical Instrument Co., Ltd. (Shanghai, China). The JY-ZY5 metastatic electrophoresis bath was from Beijing JunYi DongFang Electrophoresis Co., Ltd. (Beijing, China). The Molecular Imager Gel Doc XR/XRS system was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA), and the MCO-15AC CO_2 incubator was from Sanyo Electric Co., Ltd. (Osaka, Japan).

Treatment groups and transfection. Opti-MEM medium (225 μl/tube) was added to four Eppendorf tubes marked as tubes 1-4, and then the following solutions were added to each tube: Tube 1, 4.4 μl psiRNA-STAT3 pharmaceutical solution; tube 2, 4.4 μl Scramble plasmid solution; and tubes 3 and 4, 11 μl Lipofectamine® 2000. Following incubation for 5 min, tube 1 was mixed with 3 and tube 2 was mixed with 4, and all tubes were incubated for a further 20 min. Cultured C6 glioma cells were divided into the Mock, Scramble and psiRNA-STAT3 groups. A total of 514 μl of the appropriate plasmid solution was added to each well in the Scramble and psiRNA-STAT3 groups, while the Mock group was not treated with plasmid. Cells were transfected according to the manufacturer's protocol for Lipofectamine® 2000. The successful transfection of psiRNA-STAT3 C6 glioma cells was verified in our previous study, which resulted in mitochondrial membrane potential reduction and subsequent apoptosis (16). In the present study, the cell cycle and associated protein expression was examined.

Determination of cellular invasion ability by Transwell assay. Precooled serum-free DMEM was mixed with liquid Matrigel matrix at a ratio of 1:5, and 40 μl of this mixture was added to the upper part of Transwell chambers and incubated at 37˚C for 5 h. Serum-free DMEM (70 μl) was added to the upper part of Transwell chambers, followed by addition of 200 μl cell suspension ($4x10^5$/ml) in each well of the Transwell chambers. A total of 500 μl medium containing 30% calf serum was added to the lower chambers. All groups were incubated at 37˚C with 5% CO_2 for 8 h, and the culture solution in the upper part of the Transwell chambers was then discarded. Cells that had invaded the semipermeable membrane and attached to the lower chamber were fixed with 10% formaldehyde for 20 min at room temperature, stained with crystal violet for 15 min and then examined by inverted microscopy. Three random fields-of-view were selected, and the mean number of invading cells was calculated for each experimental group.

Determination of cell cycle distribution by flow cytometry. C6 glioma cells in the logarithmic phase were detached by digestion with 0.25% trypsin and centrifuged at 377 x g for 5 min at room temperature. The supernatant was discarded, and the cells were seeded at $1x10^6$ cells/well in 6-well plates and incubated at 37˚C with 5% CO_2 for 8 h. The cells were harvested and mixed with 80 μl each of ribonuclease, propidium iodide and Triton X-100. Following incubation at room temperature for 20 min, the cell cycle distribution was analyzed by flow cytometry.

Detection of apoptosis by flow cytometry. C6 glioma cells in the logarithmic phase were cultured and harvested as described earlier. Cells were then mixed gently with 195 μl Annexin V-FITC binding buffer, 5 μl Annexin V-FITC and 10 μl propidium iodide staining solution. Following incubation for 10 min at room temperature, cells were detected by flow cytometry to analyze the fractions of dead (quadrant P1), non-apoptotic healthy (quadrant P2), late apoptotic (quadrant P3) and early apoptotic (quadrant P4) cells.

Detection of cyclin D1, caspase-3 and Bcl-2 expression by western blotting. C6 glioma cells in the logarithmic phase were detached by digestion with 0.25% trypsin and centrifuged at room temperature and 377 x g for 5 min. The supernatant was discarded, and the cells were seeded at $1x10^6$ cells/well in 6-well plates and incubated at 37˚C with 5% CO_2 for 8 h. Next, the culture solution was discarded, RIPA lysis buffer was added to the cells, and the solution was centrifuged at 4˚C for 30 min at 15,535 x g. Protein concentration was determined with a bicinchoninic acid protein assay kit. Protein (50 μg/lane) was separated by 10% SDS-PAGE and electrotransferred to a cellulose membrane under transfer conditions of 200 mA for 90 min, followed by 0.1% Pounceau S staining for 10 min at room temperature to reversibly detect the protein bands. The stain was removed with TBST. Subsequent to blocking at 4˚C for 1 h in 5% non-fat milk in PBS buffer containing 0.1% Tween-20, the membranes were incubated with primary antibodies against cyclin D1, caspase-3, Bcl-2 and β-actin (1:300) at 4˚C overnight, followed by incubation with secondary antibodies (1:3,000) at room temperature for 1 h. Protein expression was detected by enhanced chemiluminescence reagent (Beyotime Institute of Biotechnology). Band density was analyzed with GelCapture software version 7.4.0 (DNR Bio-Imaging Systems, Ltd., Neve Yamin, Israel).

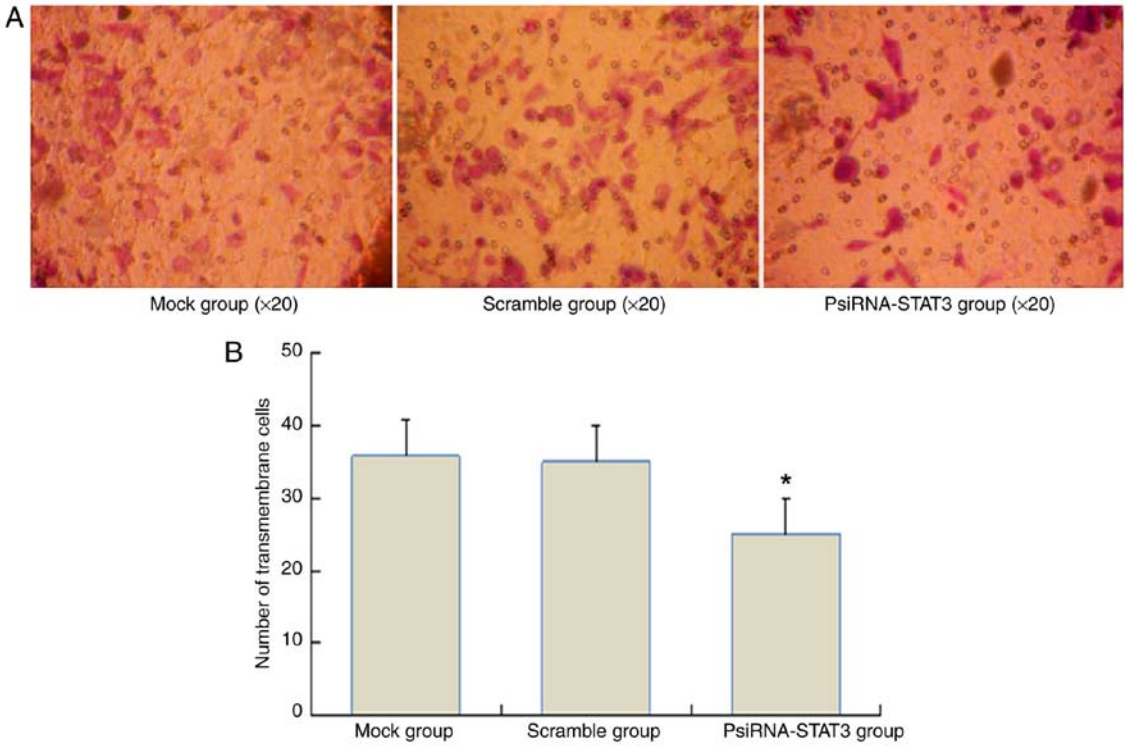

Figure 1. Effect of psiRNA-STAT3 on the invasion ability of C6 glioma cells, assessed by a Transwell assay. (A) Invasion ability of C6 glioma cells (magnification, x20). (B) Number of transmembrane cells in each group. *P<0.05 vs. Scramble and Mock groups. psiRNA, plasmid small interfering RNA; STAT3, signal transducer and activator of transcription-3.

Table I. Effects of psiRNA-STAT3 on the invasion ability of C6 glioma cells.

Group	No. of transmembrane cells
Mock	35.8±2.38
Scramble	35.0±3.08[a]
psiRNA-STAT3	25.0±3.74[b]

Data represents the mean ± standard deviation. [a]P>0.05 vs. Mock group; [b]P<0.05, vs. Scramble and Mock groups. psiRNA, plasmid small interfering RNA; STAT3, signal transducer and activator of transcription-3.

Table II. Effects of psiRNA-STAT3 on the cell cycle distribution.

	Cell cycle phase		
Group	G0/G1	S	G2/M
Mock	61.55±0.51	29.90±1.9	8.51±1.54
Scramble	61.89±0.98	30.62±0.93[a]	7.48±1.22
psiRNA-STAT3	62.64±1.5	25.73±1.87[b]	9.60±0.74

Data are presented as the mean ± standard deviation. [a]P>0.05 vs. Mock group; [b]P<0.05, vs. Scramble and Mock groups. psiRNA, plasmid small interfering RNA; STAT3, signal transducer and activator of transcription-3.

Statistical analysis. All data were collected from at least three independent experiments. Results were analyzed using SPSS statistical software (version 13.0; SPSS, Inc., Chicago, IL, USA), and are expressed as the mean ± standard deviation. Comparisons between groups were examined using Student's t-test. A P-value of <0.05 was considered to denote a statistically significant difference.

Results

Effect of psiRNA-STAT3 on invasion ability of C6 glioma cells. Compared with the Mock and Scramble groups, the number of transmembrane-invading cells was significantly reduced in the psiRNA-STAT3 group (P<0.05; Fig. 1A and B). No significant difference in the number of transmembrane cells was observed between the Scramble and Mock groups (P>0.05), indicating that psiRNA-STAT3 transfection had an inhibitory effect on the invasion ability of C6 glioma cells (Table I, and Fig. 1A and B).

Effect of psiRNA-STAT3 on the cell cycle distribution. Flow cytometric analysis revealed that the proportion of cells in S phase was markedly decreased in the psiRNA-STAT3 group compared with the Mock and Scramble groups. Furthermore, there was no significant difference among the three groups in other cell cycle phases (Table II and Fig. 2).

Effect of psiRNA-STAT3 on the apoptosis rate. As shown in Fig. 3A and B, transfection with psiRNA-STAT3 clearly promoted the apoptosis of C6 glioma cells. The cell apoptosis

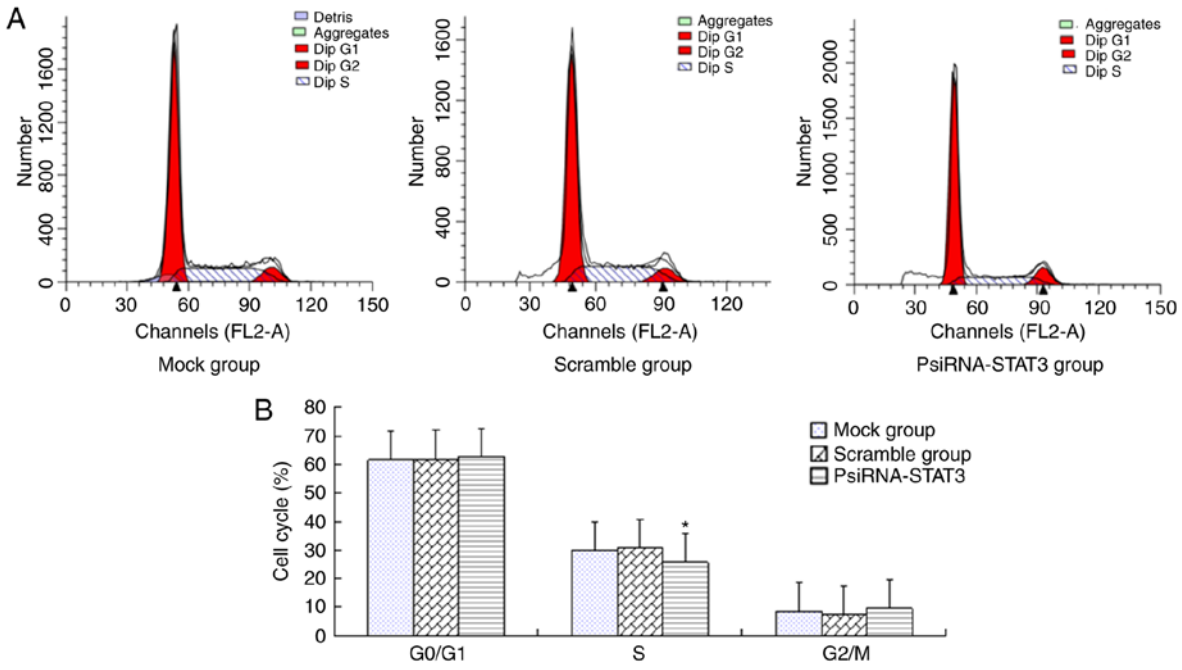

Figure 2. Effects of psiRNA-STAT3 on the cell cycle distribution. (A) Flow cytometry results, and (B) percentage of cells at each cell cycle phase. *P<0.05 vs. Scramble and Mock groups. psiRNA, plasmid small interfering RNA; STAT3, signal transducer and activator of transcription-3.

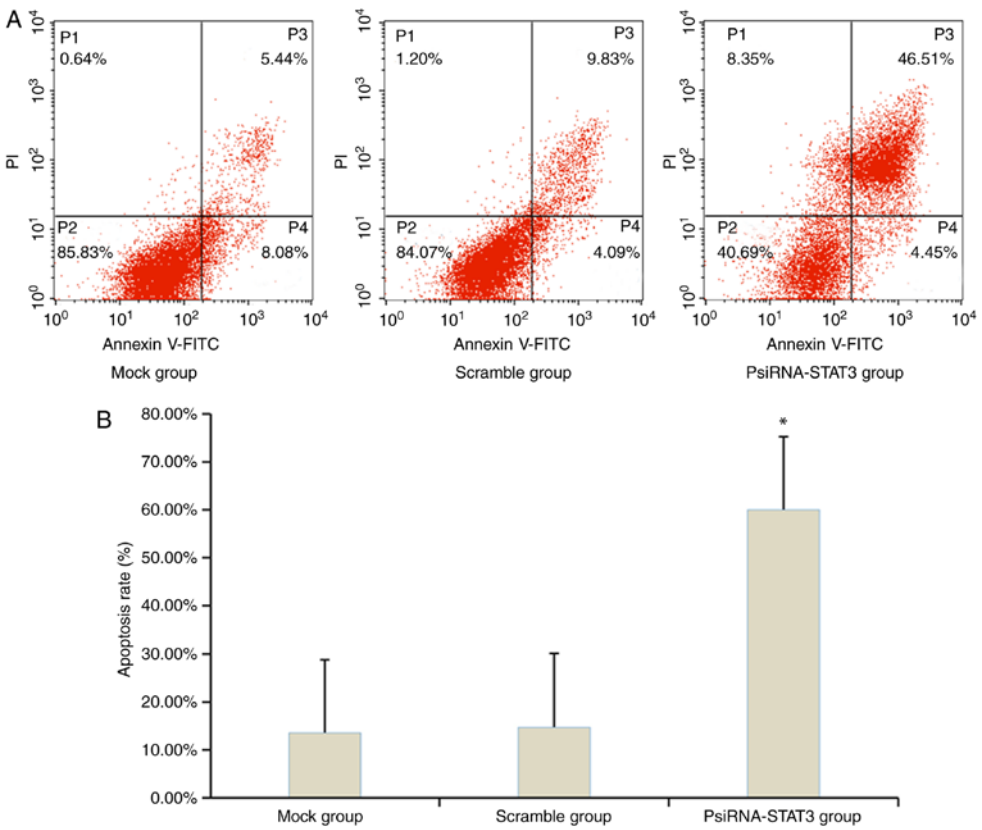

Figure 3. Effect of psiRNA-STAT3 on the apoptosis rate of C6 glioma cells, examined by flow cytometry. (A) Flow cytometry results, and (B) quantified apoptosis rate in each group. Quadrant P1 represents dead cells, quadrant P2 represents non-apoptotic healthy cells, quadrant P3 represents late apoptotic cells, quadrant P4 represents early apoptotic cells. *P<0.05 vs. Mock and Scramble groups. psiRNA, plasmid small interfering RNA; STAT3, signal transducer and activator of transcription-3.

rate was markedly increased in the psiRNA-STAT3 group compared with the Mock and Scramble groups (P<0.01), whereas no significant difference was detected between the Mock and Scramble groups. In addition, the late apoptosis rate was 5.44, 9.83 and 46.51% in the Mock, Scramble and psiRNA-STAT3 groups, respectively.

Table III. Effect of psiRNA-STAT3 on protein expression levels of cyclin D1, caspase-3 and Bcl-2 in C6 glioma cells.

Group	Grey level value		
	Cyclin D1/β-actin	Caspase-3/β-actin	Bcl-2/β-actin
Mock	0.75±0.01	1.17±0.2	0.67±0.03
Scramble	0.71±0.03[a]	0.92±0.00[a]	0.63±0.03[a]
psiRNA-STAT3	0.29±0.02[b]	0.59±0.08[b]	0.43±0.01[b]

Data are expressed as the mean ± standard deviation. [a]P<0.05 vs. psiRNA-STAT3 group. [b]P<0.05 vs. Mock group. psiRNA, plasmid small interfering RNA; STAT3, signal transducer and activator of transcription-3; Bcl-2, B-cell lymphoma-2.

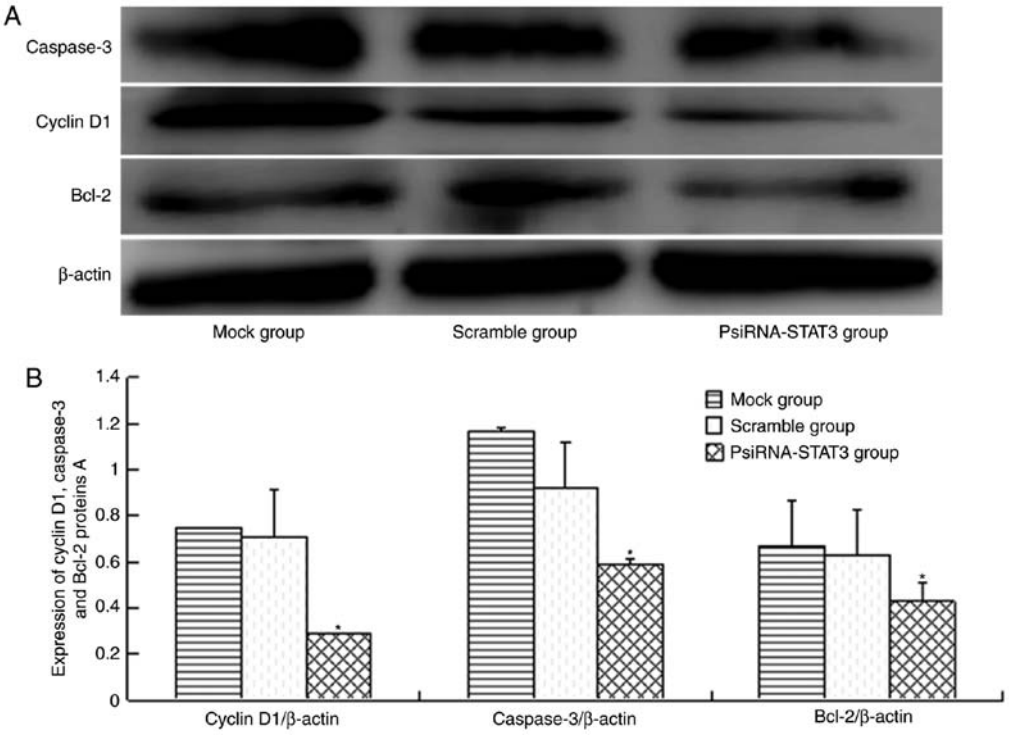

Figure 4. Effects of psiRNA-STAT3 on protein expression levels of cyclin D1, caspase-3, and Bcl-2 in C6 glioma cells. (A) Western blotting results, and (B) quantified levels of protein expression. *P<0.05 vs. psiRNA-STAT3 group. psiRNA, plasmid small interfering RNA; STAT3, signal transducer and activator of transcription-3; Bcl-2, B-cell lymphoma-2.

Effect of psiRNA-STAT3 on protein expression levels of cyclin D1, caspase-3 and Bcl-2. The expression of cyclin D1, caspase-3 and Bcl-2 was relatively high in the Mock and Scramble groups. However, the expression of cyclin D1, caspase-3 and Bcl-2 proteins decreased significantly following transfection with psiRNA-STAT3 (P<0.05). By contrast, there was no significant difference in the expression of these proteins between the Mock and Scramble groups (P>0.05). These findings suggest that psiRNA-STAT3 promoted the apoptosis of tumor cells by decreasing the expression levels of cyclin D1, caspase-3 and Bcl-2 proteins (Table III, and Fig. 4A and B).

Discussion

STAT3 is a member of the STAT family of transcription factors, which can be activated by different cytokines and growth factors. It has been reported that STAT3 has dual functions as signal transducers and transcriptional regulators, and enter the nucleus to affect gene transcription subsequent to receiving an external signal stimulus (16-18). STAT3 is of particular interest due to its close association with tumors, and there is mounting evidence that activated STAT3 serves a key role in malignant transformation (19,20). In view of this, STAT3 is considered to be a novel target for tumor treatment; therefore, specific targeting of STAT3 signaling may have therapeutic potential for malignant gliomas (21).

It is well known that tumor invasion and metastasis are important biological characteristics of malignant tumors. In the present study, the effect of psiRNA-STAT3 transfection on the migration and invasion of C6 glioma cells was investigated. The data demonstrated a significant decrease in the number of transmembrane cells in the psiRNA-STAT3 group (P<0.05) when compared with the Mock and Scramble groups,

suggesting that psiRNA-STAT3 inhibited the migration and invasion abilities of C6 glioma cells.

Cyclin D1 is regarded as a type of proto-oncogene (22) whose overexpression leads to carcinogenesis through the lack of control of cell cycle regulation, notably, the transition between G1/S phase and G2/M phase (23). Cyclin D1 overexpression shortens the G1 phase, leading to advanced S phase and hyperplasia (24,25). The data of the present study indicated that psiRNA-STAT3 altered the cell cycle progression by affecting the regulation of cyclin D1 protein expression in glioma cells. psiRNA-STAT3 treatment increased the number of cells arrested at G0/G1 phase, leading to a significant decrease in the number of cells in S phase as compared with the Mock and Scramble groups ($P<0.05$) and thus inhibiting cell proliferation. Acridine orange/ethidium bromide staining in our previous research also revealed that the proportion of cells in late apoptosis was higher in the psiRNA-STAT3 group in comparison with that in the Mock and Scramble groups (16). Furthermore, western blotting data in the present study further revealed that cyclin D1 protein expression was significantly decreased in the psiRNA-STAT3 group as compared with that in the Mock and Scramble groups ($P<0.05$).

Caspases are cysteine-containing proteolytic enzymes that are closely associated with apoptosis in eukaryotic cells, and are involved in cytokine maturation, cell growth and differentiation. The signal transduction mechanisms of apoptosis include the death receptor, mitochondrial and endoplasmic reticulum pathways. Signal transduction is initiated with the activation of caspases, and the final outcome is the cleavage of protein substrates and the breakdown of cells. Under normal conditions, caspases exist as inactive zymogens. When caspases are activated by apoptotic signals, upstream caspases sequentially activate downstream caspases in a cascade reaction and transmit apoptotic signals (26). Caspase-3 is the most important inducer of apoptosis in the caspase family, and the activation of caspase-3 can directly lead to apoptosis (27). In the present study, the results of western blotting confirmed that there was no significant difference in caspase-3 expression between the Scramble and the Mock groups, whereas caspase-3 expression was significantly decreased in the psi-STAT3 group as compared with the Mock and Scramble groups ($P<0.05$).

Bcl-2 has previously been reported to indirectly promote the occurrence and development of tumors by inhibiting glioma C6 cell apoptosis (28). Bcl-2 is one of the most important regulators of apoptosis among the Bcl-2 family, and its activation can lead to the release of cytochrome *c* via the mitochondrial pathway. This influences the activation of caspase-3, which sets off a downstream cascade reaction. In the present study, a significant decrease in the expression of the anti-apoptotic protein Bcl-2 was also observed in the psiRNA-STAT3 group in comparison with its expression in the Mock and Scramble groups ($P<0.05$). Taken together, the present study revealed that the expression levels of cyclin D1, Bcl-2 and caspase-3 were significantly reduced in the psiRNA-STAT3 group, indicating that STAT3 silencing promoted glioma cell apoptosis.

In conclusion, the results reported in the present study revealed that psiRNA-STAT3 promoted the apoptosis of glioma cells by decreasing the expression levels of cyclin D1, Bcl-2 and caspase-3. Furthermore, the expression of caspase-3 was positively associated with that of Bcl-2, and psiRNA-STAT3's mechanism of action may inhibit caspase-3 activity. This would result in a decrease in Bcl-2 expression, leading to apoptosis and inhibiting the development of glioma. These findings provide a new theoretical basis for the clinical treatment of glioma.

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Availability of data and materials

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

Authors' contributions

QZ designed and supervised the study. JC designed the study and wrote the paper. XZ, YZ, JL and FL reviewed the manuscript, analyzed the data and performed the experiments. 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 interests.

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