Knockdown of STAT3 by shRNA inhibits the growth of CAOV3 ovarian cancer cell line in vitro and in vivo

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Constitutively activated signal transducer and activator of transcription 3 (STAT3) plays an important role in the formation of many tumors including ovarian cancer. In this study, RNA interference specific to STAT3 was employed to study its effects on the inhibition of STAT3 signaling and on the growth of ovarian cancer CAOV3 cells. Plasmid vectors pGenesil-1-GFP-U6 expressing specific small hairpin RNA (shRNA) against STAT3 and the scrambled shRNA control were constructed. After transfection into CAOV3 cells, the STAT3 shRNA specifically suppressed STAT3 expression at both mRNA and protein levels. At the same time, expressions of Bcl-xL, cyclin D1, and c-myc were down-regulated, whereas the cleaved caspase 3 was up-regulated. In addition, STAT3 knockdown inhibited anchorage-independent growth and induced apoptosis in CAOV3 cells, and decreased tumor growth in nude mice implanted with ovarian cancer cells.

Keywords CAOV3; STAT3; siRNA; cell proliferation; apoptosis

Ovarian cancer is one of the leading causes of death in women with gynecological neoplasms [1]. To develop new strategies for treatment, it is pivotal for us to gain a better understanding of the molecular mechanisms of the proliferation and differentiation of ovarian cancer cells.

Signal transducers and activators of transcription (STATs) constitute a family of transcription factors involved in cellular responses to cytokines and growth factors. Previous studies revealed that the STAT pathway plays important roles in several malignant human cancers including ovarian cancer [2,3], and activated STAT signaling is involved in cell growth and differentiation. Accordingly, the disregulation in the activation of STATs is associated with malignant transformation [4]. Constitutive activation of STAT3 has been reported in a variety of cancer cells such as breast carcinoma, lymphoma, prostate cancer, and ovarian cancer cells. As STAT3 activation promotes tumorigenesis through its effects on cell proliferation, differentiation, and anti-apoptosis [2,3,5], the STAT3 signaling pathway is a potential target for tumor therapy.

RNA interference (RNAi) is a relatively new technology that has been used for sequence-specific gene silencing. It is mediated by short interfering RNA (siRNA) molecules produced from long double-stranded RNA (dsRNA) by the enzymatic activity of Dicer in cells. RNA interference can be experimentally achieved by delivery of a synthetic dsRNA or a plasmid DNA vector containing sequence coding for a small hairpin RNA (shRNA) [6].

In the present study, we showed that vector expressing STAT3-specific shRNA knocked down the expression of STAT3 in transfected CAOV3 ovarian cancer cells, and inhibited cell growth in vitro and in vivo, which might be correlated with the down-regulation of Bel-xL, cyclin D1, and c-myc, and the up-regulation of the cleaved caspase 3 in these cells.

Materials and Methods

shRNA preparation and plasmid construction

The pGenesil-1-GFP-U6 plasmid from Genesil Biotechnology (Wuhan, China) was used for DNA vector-based shRNA construction. Based on the cDNA sequence of
STAT3 (GenBank accession No. NM_003150) and the shRNA designing tool provided freely by Ambion at its website (http://www.ambion.com/techlib/misc/shRNA_finder.html), we synthesized DNA templates encoding one STAT3-specific shRNA and one scrambled missense control (Sangon Biological Engineering Technology and Services, Shanghai, China). The sequences of oligonucleotides encoding STAT3 shRNA were: 5′-GATCCCCATCTGGCTAGATCGGCTATGCTTTTCTCGACA-3′ (upper strand) and 5′-AGCTTGACACAAACAAACATCTGCGCTAGTCCGATCTAGGCGAGATG-3′ (lower strand). The sequences encoding the shRNA control were scrambled from the above STAT3 DNA: 5′-GATCCCCATCTGGCTAGATCGGCTATGCTTTTCTCGACA-3′ (upper strand) and 5′-AGCTTGACACAAACAAACATCTGCGCTAGTCCGATCTAGGCGAGATG-3′ (lower strand). The sequences encoding short hairpin DNAs containing a sense strand of 19 nucleotides followed by a short spacer (TTCAAGACG) and six Ts that act as the transcriptional stop signal. The oligonucleotide pairs were designed to form artificial enzymatic overhangs for BamHI, SalI, or HindIII at their terminals, and thus can be directly ligated into pGenesil-1-GFP-U6 after annealing. After ligation and transformation into bacteria, the positive colonies were selected by antibiotic resistance. The correct sequences of the final DNA preparations were confirmed by sequencing (Sangon).

Cell culture and transfection
The human ovarian carcinoma cell line CAOV3 was purchased from the China Center for Type Culture Collection (Wuhan, China) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in 5% CO2 incubator. Cells were detached with trypsin-EDTA solution. After washing, they were resuspended in fresh medium and used for subsequent experiments. For transfection, the CAOV3 cells were transfected with the plasmids with Lipofectamine reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions (in our system, the percentage of cells transfected with siRNA was approximately 75%). In all of the following experiments, whenever the shRNA plasmid transfected cells were used, mock transfection without any DNA and negative control transfection with shRNA missense control plasmid were always included as experimental controls.

Reverse transcription-polymerase chain reaction (RT-PCR)
Total RNA was extracted from cells with Trizol reagent (Invitrogen) 72 h after transfection. Two micrograms of total RNA was subjected to reverse transcription with an RT-PCR kit (Promega, Madison, USA). The primers (Sangon) used for subsequent PCR amplifications were: STAT3, 5′-TTGCCAGTTGTTGATCTGAC-3′ (forward) and 5′-CAGACCCAGAAGGAGAAGC-3′ (reverse) for amplification of a 318 bp product; c-myc, 5′-GGGCTTCTCAGAGGCTTGGC-3′ (forward) and 5′-CGTCCTTGGTCGATCTACA-3′ (reverse) for amplification of a 341 bp product; and β-actin, 5′-ATGTTGAGACTCTTGACAC-3′ (forward) and 5′-CAGTCACTCTGGATGGAGC-3′ (reverse) for the amplification of a 491 bp product. Amplification conditions were 95 °C for 5 min, then 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s, followed by the 72 °C extension for 3 min.

Cell proliferation assay
The viability of CAOV3 cells was determined with a 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay kit (Promega). Cells were plated in a 96-well cell culture plate at the density of 10,000 cells/well, five wells/group, then transfected as described above. Seventy-two hours after transfection, the novel tetrazolium compound MTS was added to each well with culture medium and incubated for 2 h. The absorbance of each well was measured at 490 nm with a 96-well plate reader (680 microplate reader; BioRad, Hercules, USA).

Anchorage-independent growth assay
Anchorage-independent growth of tumor cells was assayed by their ability to form colonies in agar plates. Forty-eight hours after transfection, CAOV3 cells were trypsinized and suspended in DMEM with 0.3% agar at a density of 600 cells/ml, and seeded into a 6-well plate at 1 ml/well, five wells/group, then incubated in regular culture medium at 37 °C with 5% CO2 for 2 weeks, the cells were stained with 0.3% crystal violet and colonies larger than 100 µm in diameter were counted.

Determination of DNA fragmentation
Apoptosis was assayed with a cell death detection enzyme-linked immunosorbent assay kit (Roche Diagnostics,
Lewes, UK) designed to detect apoptosis-induced DNA fragmentation. The transfected cells were cultured for 3 d and the fragmented DNAs from these cells were measured according to the manufacturer’s instructions.

**Flow cytometry analysis**
Distribution of cells at different stages of the cell cycle was analyzed by flow cytometry. Approximately $1 \times 10^6$ cells were fixed by PBS with 75% ethanol at 4 ºC for 40 min, and incubated with 40 ng/ml propidium iodide (Sigma, St. Louis, USA), 0.1 mg/ml RNase, and 0.1% Triton X-100, at room temperature for 40 min, then analyzed by flow cytometry (Becton Dickinson Immunocytometry Systems, San Jose, USA) for DNA contents.

**Western blot analysis**
Cells ($5 \times 10^3$) were washed and lysed in 100 µl of lysis buffer (20 mM HEPES, pH 7.3, 1% Triton X-100, 10% glycerol, 50 mM NaF, 0.1 mM EDTA, 10 µg/ml phenylmethylsulphonyl fluoride, 1 mM Na$_2$VO$_4$, and 5 µg/ml leupeptin) on ice for 30 min. Lysates were centrifuged at 10,000 g at 4 ºC for 10 min. Approximately 15 µl or 40 µg of total protein in the supernatant was subjected to SDS-PAGE and reacted with anti-STAT3, anti-phosphorylated-STAT3 (p-STAT3; Ser727), anti-Bcl-xL, anti-cleaved caspase 3 (Asp175), and anti-cyclin D1 antibodies (Cell Signaling Technology, Beverly, USA). Anti-β-actin antibody from Santa Cruz Biotechnology (Santa Cruz, USA) was used as the Western blot loading control.

**Tumor growth in vivo**
The male nude mice (5−6-week-old) were maintained in a temperature-controlled environment with free access to standard rodent chow and water. Experiments were carried out in accordance with the ethical guidelines of Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). Equal numbers of CAOV3 cells transfected with STAT3 or scrambled STAT3 were collected by trypsinization 3 d after transfection. The cells were washed three times with 1×PBS and resuspended in 0.1 ml saline solution. Each mouse was injected with $5 \times 10^6$ cells to form a tumor xenograft and eight mice were used in each transfected group. Then STAT3 siRNA or scrambled STAT3 vector diluted in saline were directly injected into the tumors at a dose of 20 µg every 7 d. The tumor volumes were assessed once every week for a total of 6 weeks by measuring the two perpendicular dimensions with a caliper according to the formula: volume=$(a \times b^2)/2$, where $a$ is the larger and $b$ is the smaller dimension of the tumor.

**Statistical analysis**
The data obtained were statistically evaluated using one-way ANOVA followed by Bonferroni’s test. Differences in values with $P<0.05$ were considered statistically significant.

**Results**
CAOV3 cells were transfected with DMEM alone (mock), or the plasmid expressing STAT3 scrambled shRNA (scrambled vector) or the plasmid expressing STAT3-targeted shRNA (STAT3 vector). At 72 h after transfection, they were then harvested for the extraction of total RNA or proteins, or subjected for further analysis on their growth capacities and their apoptosis, as described in detail below.

**Efficient knockdown of STAT3 by STAT3-specific shRNA**
The STAT3 mRNA levels in transfected cells were measured by semi-quantitative RT-PCR, and the protein levels for STAT3 and p-STAT3 were determined by Western blot analysis. As shown in Fig. 1(A), STAT3 mRNA expression was significantly suppressed in CAOV3 cells transfected with the STAT3 shRNA expressing vector. Accordingly, the protein levels for both STAT3 and its activated form, phosphorylated-STAT3 [Fig. 1(B)] were also markedly reduced in the same cells. In mock transfection or the scrambled shRNA control, cells showed no significant changes in the levels of STAT3 mRNA or protein. In addition, as shown in Fig. 1(C), treatment of CAOV3 cells with STAT3 shRNA caused a time-dependent inhibitory effect on STAT3 expression in CAOV3 cells.

**Inhibition of cancer cell proliferation by STAT3 knockdown**
To examine the effect of STAT3 knockdown on proliferation of CAOV3 cells, the MTS kit was used to measure proliferation status of the cells. Fig. 2 indicates that treatment of CAOV3 cells with STAT3-targeted shRNA resulted in approximately 50% growth inhibition of CAOV3 cells, whereas no significant inhibition was observed in the cells transfected with the STAT3 scrambled vector compared with the mock transfected cells.

**Inhibition of anchorage-independent growth by STAT3 knockdown**
Anchorage-independent growth in 3-D agar gel is a

Knockdown of STAT3 expression by shRNA in CAOV3 cells

measure of cellular transformation, one of the main characteristics of cancer cells. When transfected cells were plated into 3% agar plates and grown for 2 weeks, we observed a significant decrease of approximately 62% in their ability to form colonies for STAT3 knockdown CAOV3 cells compared with both controls, indicating a reduced transforming ability (Fig. 3).

**Induction of apoptosis by STAT3 knockdown**

Apoptosis induced by STAT3 shRNA in CAOV3 was examined by following the kinetics of DNA fragmentation with a nucleosomal fragment detection enzyme-linked immunosorbent assay kit. STAT3 knockdown significantly increased DNA fragmentation compared with the controls [Fig. 4(A)]. DNA content analysis by flow cytometry showed that STAT3 shRNA obviously increased the

![Fig. 1 Suppression of signal transducer and activator of transcription 3 (STAT3) expression by small hairpin RNA (shRNA) in ovarian cancer CAOV3 cells](image1)

CAOV3 cells were transfected with Dulbecco’s modified Eagle’s medium without any DNA (mock), or the STAT3 scrambled vector (scrambled vector), or the STAT3-targeted shRNA vector (STAT3 vector) and continued in culture for 72 h. (A) Total RNAs were extracted and analyzed by RT-PCR with primers specific to STAT3 and β-actin. (B) Total proteins were extracted and analyzed by Western blotting with the indicated antibodies. (C) STAT3 protein expression in the STAT3 short interfering RNA-treated CAOV3 cells at different transfection times. Images shown are representative of three independent experiments. p-STAT3, phosphorylated-STAT3.

![Fig. 2 Growth inhibition of ovarian cancer CAOV3 cells by signal transducer and activator of transcription 3 (STAT3) knockdown](image2)

CAOV3 cells were transfected with Dulbecco’s modified Eagle’s medium without any DNA (mock), or the STAT3 scrambled vector (scrambled vector), or the STAT3-targeted shRNA vector (STAT3 vector) and continued in culture for 72 h. Cell proliferations were determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt assay. Data were expressed as the mean±SD from five wells in each group. *P<0.05 compared with the scrambled vector control by Bonferroni’s test. Data shown are representative of three independent experiments.

![Fig. 3 Inhibition of anchorage-independent growth of ovarian cancer CAOV3 cells by signal transducer and activator of transcription 3 (STAT3) knockdown](image3)

CAOV3 cells were transfected with Dulbecco’s modified Eagle’s medium without any DNA (mock), or the STAT3 scrambled vector (scrambled vector), or the STAT3-targeted shRNA vector (STAT3 vector) and continued in culture for 2 d. They were then plated into 3% agar. After 2 weeks, the numbers of colonies formed were counted after crystal violet staining under a light microscope. Data are expressed as the mean±SD from five wells in each group. *P<0.05 compared with the mock transfection and the scrambled vector control by Bonferroni’s test. Data shown are representative of three independent experiments.
fraction of cells at the sub-G\(_1\) phase of the cell cycle [Fig. 4(B)]. In addition, cleaved caspase 3 was detected only in the cells treated with STAT3 shRNA by Western blot analysis [Fig. 4(C)], but not in any of the control cells.

**Down-regulation of Bcl-xL, cyclin D1, and c-myc by STAT3 knockdown**

Previous studies have shown that constitutive activation of STAT3 induces the expression of Bcl-xL, cyclin D1, and c-myc, which might contribute to tumorigenesis. We investigated the potential effects of STAT3 knockdown on these STAT3 downstream molecules. Western blot analysis detected the specific bands for Bcl-xL and cyclin D1 individually in transfected CAOV3 cells. As shown in Fig. 5(A), the protein levels of Bcl-xL and cyclin D1 were significantly reduced in STAT3 knockdown CAOV3 cells. RT-PCR analysis revealed that c-myc mRNA was also decreased [Fig. 5(B)] in the same cells.

**Inhibition of in vivo tumor growth by STAT3 knockdown**

To investigate the potential effect of STAT3 knockdown on the growth of ovarian cancer cells in vivo, ovarian tumor xenografts were produced in nude mice with STAT3 knockdown CAOV3 cells. CAOV3 cells were transfected with Dulbecco’s modified Eagle’s medium without any DNA (mock), or the STAT3 scrambled vector (scrambled vector), or the STAT3-targeted shRNA vector (STAT3 vector) and continued in culture for 72 h. (A) Total proteins were extracted from cells and analyzed by Western blotting with antibodies against Bcl-xL and cyclin D1. (B) Total RNAs were extracted and analyzed by RT-PCR with primers specific to c-myc and \(\beta\)-actin. Data shown are representative of three independent experiments.
Knockdown of STAT3 expression by shRNA in CAOV3 cells

knockdown CAOV3 cells or the controls. Growth curves of these xenografts over 6 weeks showed that the STAT3 shRNA group displayed a significantly lower growth rate, only approximately 50% of that obtained from the two control groups (Fig. 6).

Discussion

STAT3 exists in a latent form in the cytoplasm and can be activated by a range of signaling pathways starting from the activation of many cell surface receptors by tyrosine phosphorylation. This mechanism of STAT3 activation has been shown to be involved in the elevated levels of cell proliferation, anti-apoptotic effects, and cell cycle progression in many cancer cells [7]. STAT3 protein contains a tetramerization and a leucine zipper domain at its N-terminus, a DNA-binding domain in the middle, and an SH2 transactivation domain at the C-terminus [8].

Upstream regulators of STATs, such as Janus family tyrosine kinases (JAKs), steroid receptor co-activator (Src) and epidermal growth factor (EGF), regulate STAT3 by phosphorylation at Tyr705, which in turn results in the activation of a cascade of STAT3-mediated signaling events [9]. Under physiological conditions, STAT3 activation is transient and lasts from several minutes to several hours, so aberrantly sustained activation of STAT3 could lead to uncontrolled growth and prolonged survival often seen in cancer cells [10].

RNAi technology is widely used in gene therapy and as a research tool for gene modulations because it allows the selective, transient knockdown of targeted protein expression. In mammalian cells, RNAi can be triggered by siRNAs that cause strong yet transient inhibition of the expression of specific genes. This could be achieved by delivering a synthetic double-stranded siRNA or a plasmid vector containing DNA sequence coding for an shRNA. Chemically synthesized siRNAs can be delivered to the cytosol and directly combined with RNAi silencing complex, resulting in effective suppression of gene expression. However, such suppression is transient, as the oligos are easily degraded by the host cell. The expression of shRNA from plasmid has some advantages as shRNA plasmids can repeatedly transcribe similar dsRNA products and suppress gene expression over a longer period of time. This plasmid vector-based approach is also less expensive compared with the direct delivery of the synthesized siRNA [11,12]. shRNA vectors have been shown to efficiently block expression of specific proteins both in vitro and in vivo, and these artificial shRNAs are transcribed from the U6 or H1 promoters in the targeting vectors [13].

The effect of STAT3 knockdown in ovarian cancer has not been well studied. Here, we showed that transfection into CAOV3 cells with STAT3 shRNA vector dramatically inhibited the expression of STAT3 mRNA as well as protein, and also decreased cell proliferation and anchorage-independent colony formation. Combined analyses of DNA fragmentation, cell cycle progression by flow cytometry, and cleaved caspase 3 expression by Western blot analysis indicated that STAT3 knockdown induced apoptosis of CAOV3 cells. Importantly, STAT3 shRNA also significantly suppressed tumor cell growth in vivo.

Previous studies showed that constitutively activated STAT3 led to up-regulation of its downstream molecules such as anti-apoptotic protein Bcl-2 and cell cycle regulators cyclin D1 and c-myc, all playing important roles in oncogenesis [14,15]. Consistent with these reports, our study showed that CAOV3 cells with STAT3 knockdown decreased their expression of Bcl-xL protein, as well as the expression of cyclin D1 and c-myc [Fig. 5(A)]. This might imply that STAT3 plays a role in the regulation of these molecules in ovarian cancer cells, and its knockdown results in growth inhibition and induction of...
apoptosis.

In summary, our study showed that knocking down STAT3 with shRNA produced from pGenesil-1-GFP-U6 led to growth inhibition and apoptosis of CAOV3 cells in vitro and inhibition of tumor growth in vivo. Therefore, it is tempting to speculate that STAT3 signaling might play a considerable role in ovarian cancer, in addition to its known roles in many other cancers.

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Knockdown of STAT3 expression by shRNA in CAOV3 cells