Effects and mechanism of STAT3 silencing on the growth and apoptosis of colorectal cancer cells

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Abstract. Signal transducer and activator of transcription 3 (STAT3) have roles in various cellular processes, including angiogenesis, apoptosis, cell cycle progression, cell migration and drug resistance. To clarify the effects of STAT3 in colorectal cancer (CRC) cells and the underlying molecular mechanisms, STAT3 was directly silenced, and the effects of STAT3 silencing on cell proliferation, apoptosis and growth with phenotype-associated molecules were examined. pSH1-Si-STAT3 was successfully transfected into the CRC HCT-116 and SW480 cell lines, which was verified by GFP tagging under a fluorescence microscope. An MTT assay revealed that the proliferation of both cell lines that were transfected with pSH1-Si-STAT3 was significantly suppressed in comparison with the control and mock (P<0.05). Acridine orange/ethidium bromide staining and flow cytometry indicated that the transfected cell lines had a significantly higher rate of apoptosis than the control- and mock-treated cells (P<0.05). STAT3-silienced cells were also significantly arrested at the G_2/M stage compared with the cells that were transfected with control and mock plasmids (P<0.05). At the mRNA level, the expression of STAT3 and survivin was significantly downregulated (P<0.05), but p53 and caspase-3 were significantly upregulated (P<0.05). The significantly different patterns of expression were observed in western blot analysis (P<0.05). The findings of the present study indicate that STAT3 silencing may suppress the proliferation and growth of CRC cells, and induce their apoptosis by upregulating the expression of survivin, p53 and caspase-3. Therefore, STAT3 may be a good candidate for CRC gene therapy.

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Introduction

Signal transducer and activator of transcription 3 (STAT3) is implicated in angiogenesis, apoptosis, cell-cycle progression, cell migration and drug resistance by regulating the expression of phenotype-associated genes (1,2). Canonical STAT3-mediated signaling involves the phosphorylation of specific tyrosine residues that leads to homodimerization and translocation to the nucleus upon the stimulation of cytokines and growth factors (3,4). Nuclear STAT3 fine-tunes autophagy by regulating the transcriptional expression of autophagy-associated genes and autophagy-targeting microRNAs, whereas its cytoplasmic counterpart constitutively suppresses autophagy by sequestering eukaryotic translation initiation factor 2-α kinase 2 or interacting with forkhead box O (FOXO)1 and FOXO3 (4,5). Additionally, STAT3 promotes mitochondrial transcription and oxidative respiration. The mitochondrial translocation of STAT3 serves a major role in IgE-antigen-mediated mast cell exocytosis and optimal electron transport chain activity, and protects against oxidative-stress-induced mitochondrial dysfunction and autophagy (6-8).

Although the levels of activated STAT3 remain transient in normal cells, it is overactive in ~70% of human solid tumors and regulates the expression of numerous oncogenic genes that control the growth and metastasis of tumor cells (3). A meta-analysis revealed that elevated phosphorylated (p)-STAT3 levels are significantly associated with tumor cell differentiation, lymph node metastases and poor overall and disease-free survival rates inpatients with cancer of the digestive system (9). Janus kinase (JAK)-STAT3 signaling promotes oncogenesis by upregulating the proliferation, survival, invasion of tumor cells and immunosuppression (10). In recent years, antitumor approaches targeting STAT3 activity include the inhibition of tyrosine kinases or STAT3 dimer formation (11,12). The present study directly silenced STAT3 and observed the effects on the proliferation and growth of colorectal cancer (CRC) cells. Additionally, the associated molecular mechanisms were also clarified.

Materials and methods

Cell culture and transfection. HCT-116 and SW480 cells were obtained from the Cell Resource Center, Institute of Basic Medicine, Peking Union Medical University (Beijing, China) and grown in a humidified atmosphere with 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Corning Incorporated, Corning, NY, USA). Penicillin (100 U/ml) and streptomycin (100 μg/ml) were added to the medium. All cells were collected by trypsinization at 37°C followed by centrifugation (3,000 x g, 5 min, room temperature), washed in phosphate buffered saline (PBS) and subjected to total RNA and protein extraction.

Either $1.0x10^6$ HCT-116 or SW480 cells were transfected with pSilencer3.0-H1-STAT3-siRNA-GFP at 500 ng/μl (pSH1Si-STAT3 group) or pSH1Si-Scramble at 500 ng/μl (mock group) following seeding on dishes according to the manufacturer's instructions of Attractene Transfection Reagent (301005; Qiagen, Inc., Valencia, CA, USA). The untreated cells were used as control. After 2 h of transfection, cells were selected by G418 with the collection of monoclones, and immediately used for following proliferation assay, Acridine orange (AO)/ethidium bromide (EB) staining, PI staining and animal experiments were performed following transfection. The shRNA targets STAT3 nucleotides 2144-2162 (GenBank accession no. NM00315): Forward, 5'-GCAGCAGCTGAACAACAT GTTCAAGAGACATGTTGTTCAGCTGCTGCTTTTT-3' and reverse, 5'-AATTAAAAAGCAGCAGCTGAACAACATGTC TCTTGAACATGTTGTTCAGCTGCTGCTGCGGCC-3'.

Proliferation assay. HCT-116, SW480 and pSH1Si-STAT3-transfected cells were seeded at $2.0x10^3$ cells per well in 96-well plates and maintained in media containing 10% FBS. At 0, 24, 48 and 72 h, the cells then were incubated with 20 μl MTT (5 mg/ml) for 4 h at 37°C. The medium was then discarded and 150 μl dimethyl sulfoxide was added to each well to dissolve the precipitates. The number of viable cells was counted by measuring the absorbance at 490 nm using a microplate reader. The experiment was repeated three times.

AO/EB staining. AO is a nucleic-acid-selective fluorescent cationic dye that can pass through live and dead cells. Upon treatment with the two reagents, live cells appear uniformly green under a fluorescence microscope, whereas apoptotic cells stain green and bright green dots appear in the nuclei. Necrotic cells stain orange under a fluorescence microscope. EB can only stain cells that have lost membrane integrity. In brief, HCT-116, SW480, pSH1Si-Scramble- and pSH1Si-STAT3-transfected cells were resuspended in an AO solution at $1x10^{10}$ cells/l. A total of 2 μl AO solution (100 mg/ml) and 2 μl EB solution (100 mg/ml) were added into 20 μl cells for 5 min at room temperature. Next, the cells were seeded onto glass slides and observed in 5 fields under a fluorescence microscope (x200 magnification).

Flow cytometry. HCT-116 and SW480, pSH1Si-Scramble- and pSH1Si-STAT3-transfected cells were washed twice with PBS then detached by trypsinization with free EDTA. The cells were collected in a centrifuge tube and incubated with 1 ml precooled 70% ethanol for ≥12 h. The cells were washed twice with PBS again and incubated for 1 h at 37°C with 1 ml RNase (0.25 mg/ml, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The cells were resuspended in propidium iodide (PI) at a concentration of 50 μg/ml, and cell cycle analysis was performed using a flow cytometer with CytExpert software (version 2.0; Beckman Coulter, Inc., Brea, CA, USA) used for analysis.

Xenograft models. A total of 15 male BALB/c nude mice of 6-8 weeks (17.76±1.83 g) were kept in a specific pathogen-free facility in a temperature-controlled animal room (22-24°C) with a 12/12 h light/dark illumination cycle. All of the mice had access to standard rodent food (Beijing Huafukang Biotech Co., Ltd., Beijing, China) and water *ad libitum*. All animal treatments were performed in accordance with international ethics guidelines and the National Institutes of Health Care and Use of Laboratory Animals. This study was approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Jinzhou Medical University (Jinzhou, China).

HCT-116, pSH1Si-Scramble- and pSH1Si-STAT3-transfected cells were grown in the medium as aforementioned. The cells were detached by trypsinization, and subsequently washed and resuspended in DMEM medium. Subcutaneous xenografts were established by injection of $1.0x10^7$ HCT-116 cells/mouse (n=5/group), and the mice were assessed every 4 days for tumor size and body weight. The model was established successfully when there were nodules with the size of a grain of rice after 1week and a volume of 50-70 mm^3 after 2 weeks.

The nude mice were divided into control, mock and treatment groups, and each group consisted of 5 mice. The treatment volume was 40 μl, and the plasmid concentration was 0.5 μg/μl, which was injected intratumorally at several points. At 30 sec after injection, an electrical impulse was applied twice at intervals of >1 min. Electrodes were placed at each end of the tumor's long and short axes (electric field strength, 200 V/cm; electric pulse duration, 50 msec and pulse frequency, 1Hz). This process was repeated on days 10 and 17. Tumor volume and mouse body weight were measured every 4 days. On day 20, the tumors were removed, and the tumor weight and volume were measured. Tumor growth was calculated as follows: Length x $width^2$ x 0.52. At the end of the experiment, the mice from each group were anesthetized, their images were captured and the mice were sacrificed. The tumors were weighed and subjected to RNA or protein extraction for subsequent experiments.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from CRC cells or tissues using TRIzol (Takara Bio, Inc., Otsu, Japan) and quantified using a NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE USA). RT-PCR was performed with 2 μg total RNA using AMV reverse transcriptase and random primers at 42°C for 1 h (Takara Bio, Inc.). PCR primers were designed and are listed in Table I. The amplification of cDNA was performed using the Hot Start Taq Polymerase kit (Takara Bio, Inc.) with GAPDH as an internal control. The PCR conditions were as follows: Denaturation

Table I. Primers used for reverse transcription-semi-quantitative polymerase chain reaction.

Gene	Primer sequence	AT (°C)	Product size (bp)	Extension time (sec)
STAT3		55	315	30
Forward	5'-TTGCCAGTTGTGGTGATC-3'			
Reverse	5'-AGACCCAGAAGGAGCCGC-3'			
Survivin		58	412	30
Forward	5'GAATTCATGGGTGCCCCGACCTTGCC-3'			
Reverse	5'-AGATCTTTCTTCTTATTGTTGGTTTCC-3'			
Caspase-3		55	241	30
Forward	5'-AGAACTGGACTGTGGCATTG-3'			
Reverse	5'-TTCTGTTGCCACCTTTCG-3'			
β-actin				
Forward	5'-AAGTACTCCGTGTGGATCGG-3'			
Reverse	5'-ATGCTATCACCTCCCCTGTG-3'	55	600	30

AT, annealing temperature; STAT3, signal transducer and activator of transcription 3.

at 95°C for 10 min; followed by 30 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec; and as a termination step, the extension time of the last cycle was increased to 7 min. The amplicons were electrophoresed in 2% agarose and visualized under UV light. Densitometric quantification was performed with β-actin as a control using ImageJ version 1.48 (National Institutes of Health, Bethesda, MD, USA). The value of the control was set to 1.

Western blot analysis. Protein extraction was performed from gastric carcinoma and epithelial cells or carcinoma tissues by sonication or homogenization in radioimmunoprecipitation assay lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 (Sigma-Aldrich; Merck KGaA), 5 mM dithiothreitol, 10 mM NaF and protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan)]. A total of 40 μg protein was measured and calculated according to Kaumas brilliant blue method (13) from HCT-116, SW480 and pSH1Si-STAT3-transfected cells, and tumors of HCT-116 and pSH1Si-STAT3-transfected cells per lane. The protein samples were resolved using 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skimmed milk in Tris-buffered saline with Tween 20 (TBST; 10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, and the primary antibodies were placed on a shaker at 4°C overnight. The antibodies against β-actin (sc-47778; 1:1,000), p-STAT3 (sc-56747; 1:500), STAT3 (sc-293151; 1:500), surviving (sc-101433; 1:500), tumor protein p53 (sc-47698; 1:500) and caspase-3 (sc-136219; 1:500) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The cells were rinsed with TBST and incubated with IgG antibody that was conjugated to horseradish peroxidase (HRP; Dako, Carpinteria CA, USA) at a dilution of 1:1,000 at room temperature for 1 h. The bands were visualized using LAS4010 (GE Healthcare Life Sciences, Little Chalfont, USA) and ECL-Plus detection reagents (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The experiment was repeated for

three times. Densitometric quantification was performed with β-actin as a control using ImageJ version 1.48. The control was considered to have a value of '1'.

Statistical analysis. The results are expressed as mean ± standard deviation. Statistical evaluation was performed using Kruskal-Wallis to compare the means of different groups. P<0.05 was considered to indicate statistical significance. SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA) was used to analyze all data.

Results

Effects and associated mechanisms of STAT3 silencing on the proliferation and apoptosis of CRC cells. pSH1-Si-STAT3 and pSH1Si-Scramble were successfully transfected into CRC cells as indicated by the findings from fluorescence microscopy (Fig. 1A). The effect of STAT3 silencing on the viability of HCT-116 and SW480 cells was assessed using an MTT assay. As shown in Fig. 1B, the proliferation of the two cell lines transfected with the pSH1-siRNA-STAT3 plasmid was significantly suppressed 48 h after transfection compared with the control and pSH1Si-scramble groups (P<0.05). There was a significantly higher rate of apoptosis in CRC cells that were treated with the pSH1-siRNA-STAT3 plasmids than those in the control and mock groups as indicated by AO/EB and PI staining (P<0.05; Fig. 1C and D). Cell cycle analysis revealed a significantly higher proportion of STAT3-silenced cells in G$_2$/M-phase arrest compared with the control and mock groups (P<0.05; Fig. 1E and F). The mRNA levels of STAT3 and survivin were significantly decreased in all cells and tumor tissues, but the expression of p53 and caspase-3 mRNA was significantly increased in the pSH1-siRNA-STAT3 group, compared with the control and mock plasmid groups (P<0.05; Fig. 1G). Western blot analysis revealed that the expression of p-STAT3, STAT3 and survivin was significantly lower in cells that were transfected with pSH1-siRNA-STAT3, compared with the control- and mock
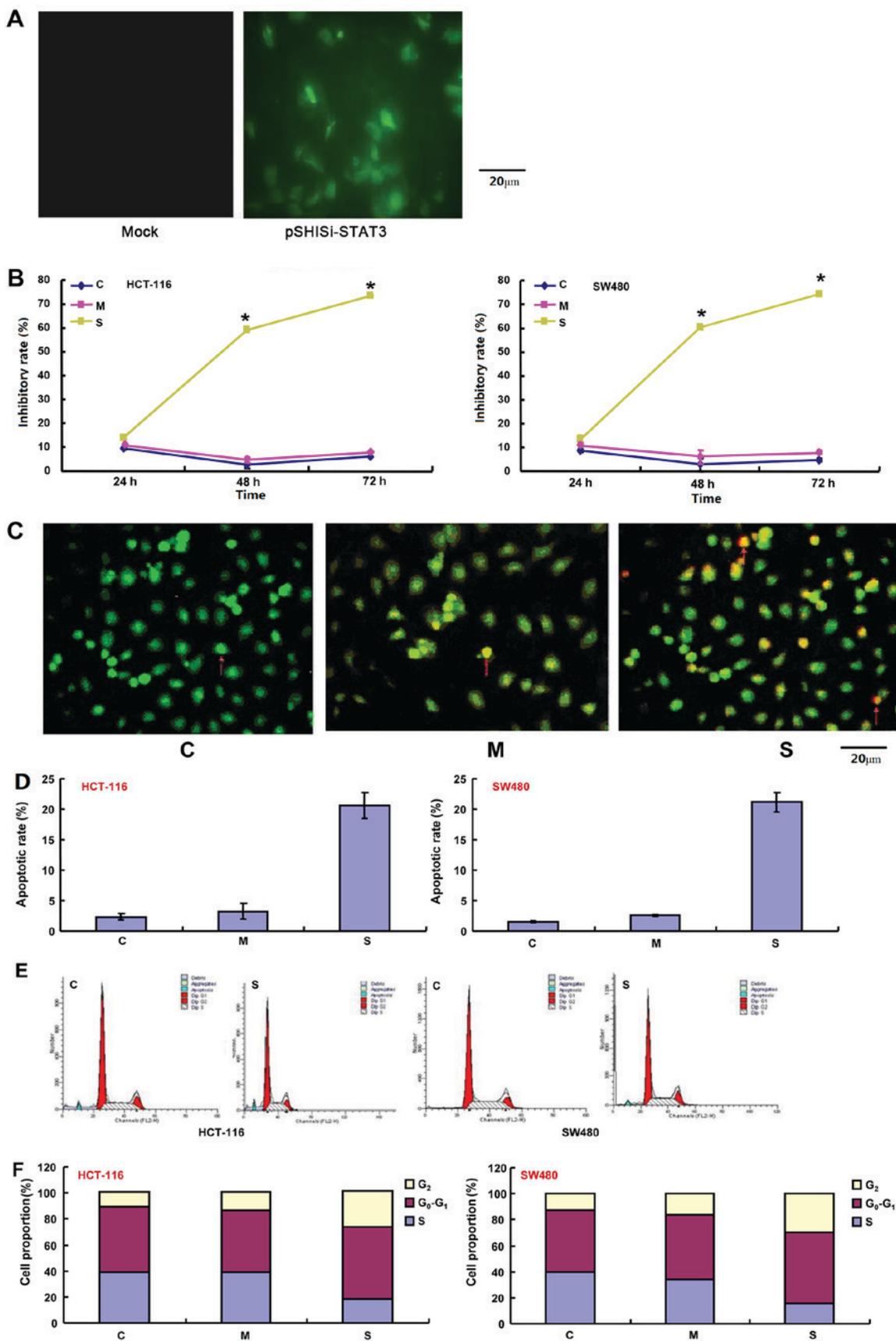
Figure 1. Effects and associated mechanisms of STAT3 silencing on proliferation and apoptosis of colorectal cancer cells. (A) Following transfection of pSH1Si-STAT3, GFP expression was observed under a fluorescence microscope. (B) The cells that were transfected with pSH1Si-STAT3 exhibited a lower growth rate compared with the control and mock cells as assessed by MTT assay. There appeared to be a higher degree of apoptosis in cells that were transfected with the pSH1Si-STAT3 plasmid than the control and mock cells as indicated by (C) acridine orange/ethidium bromide and (D) statistical analysis of apoptotic number. STAT3 silencing could induce G_2/M arrest of HCT-116 and SW480 transfectants as indicated by (E) PI staining and (F) statistical analysis of cell proportion. *$P<0.05$ compared with the control and mock
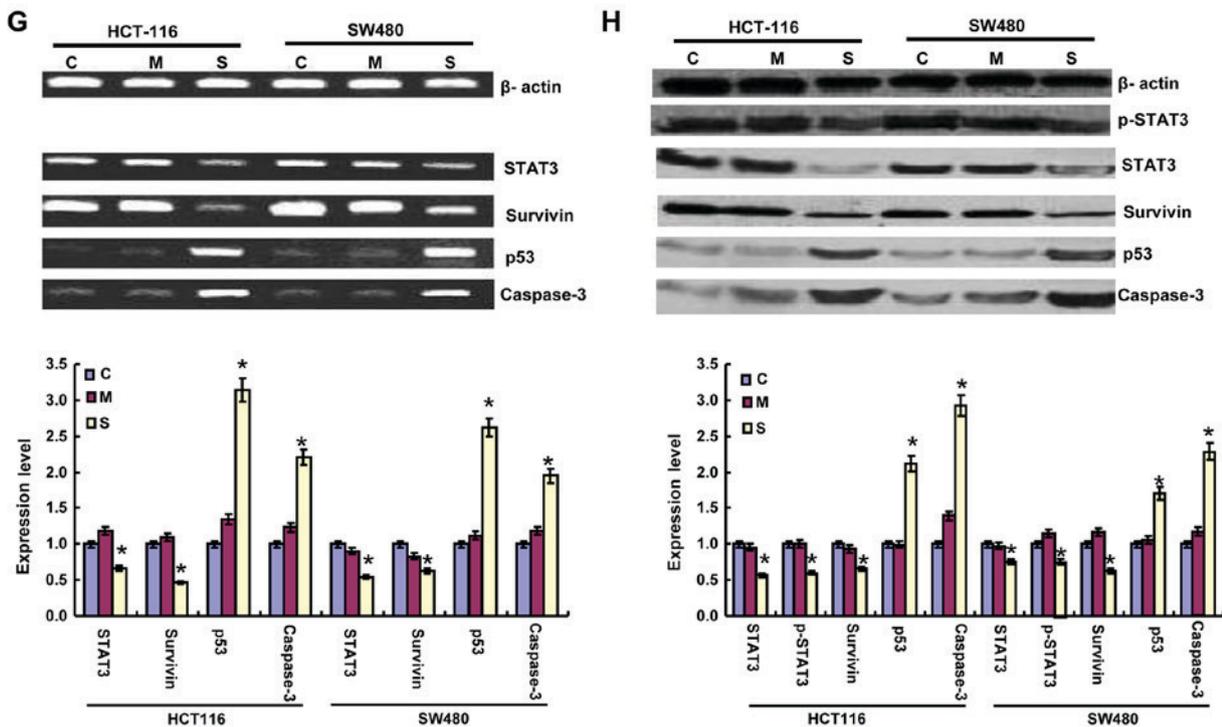
Figure 1. Continued. The phenotype-associated molecules were screened by (G) reverse-transcription-semi-quantitative polymerase chain reaction and (H) western blot analysis. *P<0.05 compared with the control or mock. p-, phosphorylated; STAT3, signal transducer and activator of transcription 3; C, control; M, mock (pSH1Si-Scramble); S, pSH1Si-STAT3; *P<0.05, compared with control or mock.

plasmid-transfected groups, but the opposite was true for p53 and caspase-3 (P<0.05; Fig. 1H).

Effects and associated mechanisms of STAT3 silencing on tumor growth of CRC cells. The volume and weight of the mouse tumors in the pSH1-siRNA-STAT3 group were significantly smaller and lower compared with the control- and mock plasmid-transfected groups (P<0.05; Fig. 2A-C). The longest diameter of a single subcutaneous tumor was 11.2 mm in the present study. The mRNA levels of STAT3 and survivin were significantly decreased in all cells and tumor tissues, but the expression of p53 and caspase-3 mRNA was significantly increased in the pSH1-siRNA-STAT3 group, compared with the control and mock plasmid groups (P<0.05; Fig. 2D). Western blot analysis revealed that the expression of p-STAT3, STAT3 and survivin was significantly lower in cells that were transfected with pSH1-siRNA-STAT3, compared with the control- and mock plasmid-transfected groups, but the opposite was true for p53 and caspase-3 (P<0.05; Fig. 2E).

Discussion

Increased STAT3 activity is common in human malignancies, including CRC. Reportedly, p-STAT3 immunoreactivity gradually increases from normal mucosa to adenoma to adenocarcinoma. p-STAT3 immunoreactivity is positively associated with the T stage and clinical stage of CRC. In a previous study involving the analysis of 47 invasive colorectal adenocarcinoma samples, STAT3 mRNA expression was significantly associated with tumor size, lymph node and metastasis stage and clinical stage of the disease (9). In CRC, nuclear p-STAT3 protein expression was associated with

the expression of its target genes B-cell lymphoma-extra large (Bcl-xL) and survivin as well as Ki-67. The inactive (UP-STAT3) and active (p-STAT3) STAT3 proteins are markedly increased in invasive CRC cases. This increase is associated with Bcl-xL and survivin induction, increased proliferation and lymph node metastasis (9,14,15). These findings indicate that STAT3 activation contributes to colorectal carcinogenesis and subsequent disease progression. In the present study, STAT3 knockdown significantly suppressed cell proliferation and tumor growth, and induced apoptosis and G_2/M arrest in HCT-116 and SW480 cells, suggesting that STAT3 could be employed as a potential target of gene therapy for patients with CRC.

Hypoxia-inducible factor 3α1 and miR-139-5p deletion promote the growth of CRC cells by activating the JAK-STAT3 signaling pathway (16,17). The insulin-like growth factor/STAT3/NANOG/Slug and interleukin-6/STAT3/Fos-related antigen-1 signaling axes simultaneously control epithelial-mesenchymal transition and maintenance of stemness in CRC, which exhibit drug resistance, invasion and metastasis (18,19). It was documented that STAT3 inhibition sensitized CRC to chemoradiotherapy *in vitro* and *in vivo*. Furthermore, the overexpression of STAT3 was associated with the resistance of CRC cells to 5-fluorouracil-based chemoradiotherapy (20,21). The inhibition of DNA methyltransferase by miR-124 and miR-874 was demonstrated to suppress growth and induce apoptosis in CRC by targeting STAT3, where STAT3 is often hyper-activated (22-24). The overexpression of B7-H3 was able to induce resistance to apoptosis in CRC cell lines by upregulating the Jak2-STAT3 signaling pathway, whereas suppressor of cytokine signaling 3 served as a negative regulator of the JAK-STAT3 pathway to
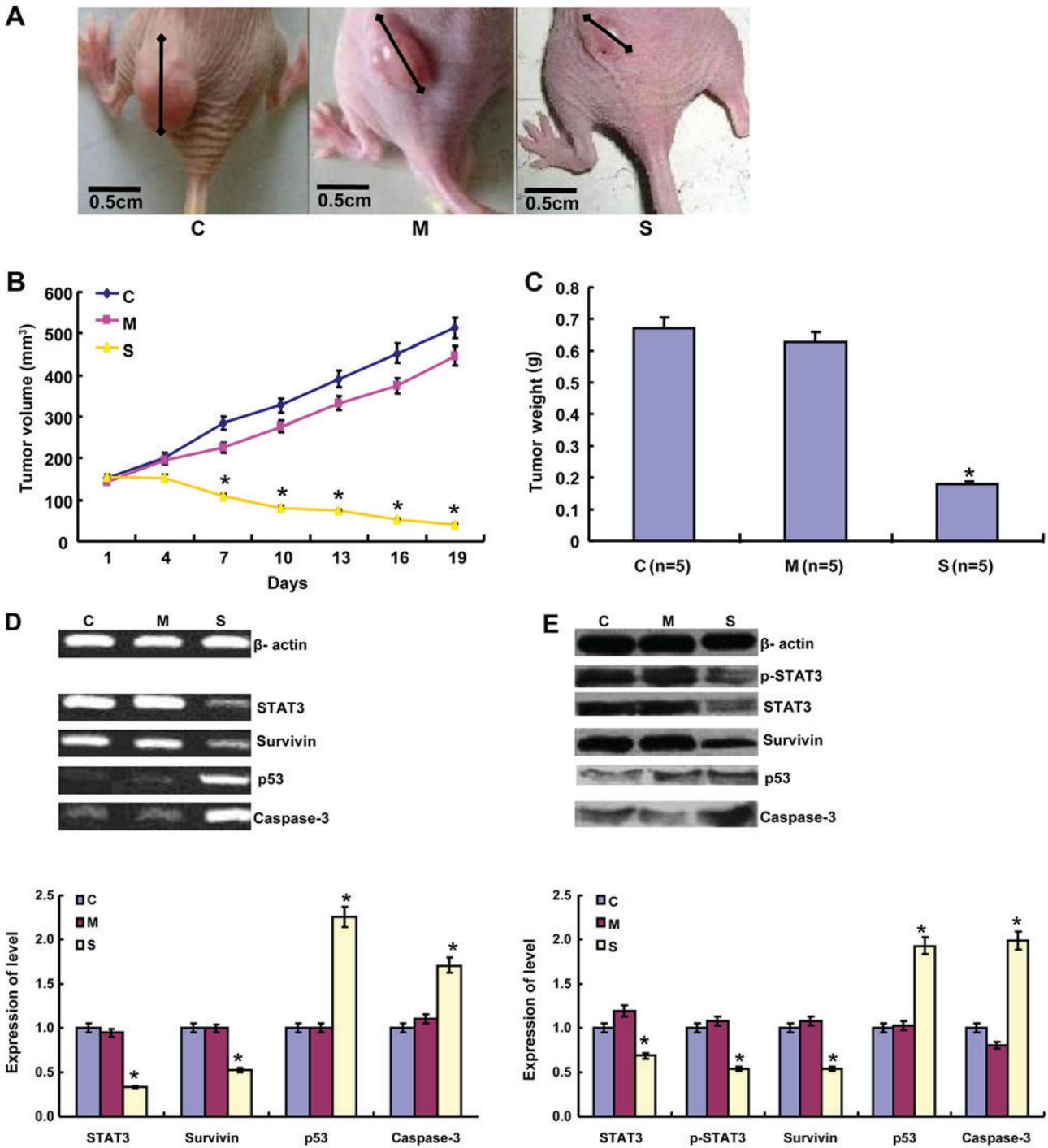
Figure 2. Effects and associated mechanisms of STAT3 silencing on tumor growth of colorectal cancer cells. (A) There existed tumor in the back of nude mice in control, mock and STAT3-knockdown groups. (B) The growth of HCT-116 and mock cells was faster than pSH1Si-STAT3 tranfectants as shown by tumor volume (C). The tumor weight was lighter in STAT3-knockdown group compared with the control and mock groups. (D) Western blotting analysis and (E) reverse transcription-semi-quantitative polymerase chain reaction were performed to detect the phenotype-related molecules. *P<0.05, compared with the control or mock-transfected tumor cells. p-, phosphorylated; STAT3, signal transducer and activator of transcription 3; C, control; M, mock-pSH1Si-Scramble; S, pSH1Si-STAT3; *P<0.05, compared with control and mock.

suppress cell proliferation and cell cycle (25-27). In addition, the epidermal growth factor/phosphoinositide-3 kinase/STAT3 signaling pathway enhanced the expression of angiogenic regulators, including vascular endothelial growth factor and leptin. The inhibition of STAT3 was able to suppress angiogenesis in CRC (28,29). These data underlie the molecular basis of gene and drug therapies targeting STAT3.

In the present study, following STAT3 knockdown, the levels of p-STAT3 and STAT3 were downregulated in HCT-116 and SW480 cells or tumors, indicating that the transfections were effective. To clarify the molecular mechanisms, the expression of survivin, p53 and caspase-3 at mRNA and protein levels was detected. The silencing of STAT3 was able to markedly decrease survivin expression, but the expression

of p53 and caspase-3 was increased. Survivin is an oncogene that has been implicated in several types of human cancer. Survivin protein ameliorates caspase activation to negatively regulate apoptosis or programmed cell death and localizes to the mitotic spindle by interacting with tubulin during mitosis to modulate mitosis (30). Caspase-3 is activated in apoptotic cells by extrinsic (death ligands) and intrinsic (mitochondrial) pathways, which results in the cleavage of poly (ADP-ribose) polymerase, actin and the sterol regulatory element binding protein (31). p53 protects against the development of cancer, including the induction of apoptosis, cell cycle arrest and the maintenance of genetic stability (32). Taking the results of the present study together, it was concluded that STAT3 promoted the proliferation and growth of CRC cells and suppressed apoptosis by targeting the expression of survivin, p53 and caspase-3 and could therefore be considered to be a potential target for gene therapy.

In conclusion, the silencing expression of STAT3 *in vitro* and *in vivo* may have a therapeutic effect in CRC cells, which brings hope of clinical therapy using RNA interference via oligonucleotide drugs. Future study will continue to investigate the synergistic effects of STAT3-knockdown and other therapeutic approaches to inhibit the proliferation and induce the apoptosis of CRC cells.

Availability of data and materials

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

Authors' contributions

JL, XFY, DFS, YYL, HZS, KQH and HCZ designed the study, performed the experiment and statistical analysis. JL, KQH and HCZ prepared reviewed the manuscript. All the authors read the manuscript, took responsibility for all aspects of the work and approved the manuscript published without conflict of interest.

Ethics approval and consent to participate

The ethics of animal performance protocol was approved by Institutional Animal Care and Use Committee of The First Affiliated Hospital of Jinzhou Medical University (Jinzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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