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Original Research

Overexpression of SOCS4 inhibits proliferation and migration of cervical cancer cells by regulating JAK1/STAT3 signaling pathway

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Purpose: To delve into the related molecular mechanism of suppressor of cytokine signaling 4 (SOCS4) on cervical cancer cell proliferation and migration. *Methods*: Quantitative real-time polymerase chain reaction and western blot assays were employed to examine SOCS4 mRNA or protein expression in four human cervical cancer cell line (HeLa, SW-732, AV3, and CaSki) and normal cervical epithelium immortalized cell line (H8) MTT cell viability assays were applied to verify the cell proliferation of HeLa after overexpression of SOCS4. Wound scratch healing assays and transwell assays were applied to examine cell migration and invasion of HeLa after overexpression of SOCS4. Flow cytometry and western blot assays were applied to check the role of SOCS4 in the apoptosis of cervical cancer cells. The western blot assays were applied to examine the protein expression of JAK1, p-JAK1, STAT3, and p-STAT3 in HeLa after overexpression of SOCS4. *Results*: In this study, the results revealed that the mRNA and protein expression level of SOCS4 was lower in four human cervical cancer cell line than normal cervical epithelium immortalized cell line, respectively. Overexpression of SOCS4 inhibited the proliferation migration, and invasion of cervical cancer cells as well as promotes apoptosis of cervical cancer cells. Meanwhile, overexpression of SOCS4 in HeLa would inhibit phosphorylation of JAK1 and STAT3 protein. *Conclusion*: SOCS4 inhibited the proliferation and migration of cervical cancer cells by regulating the JAK1/STAT3 pathway.

Keywords

SOCS4; Cervical cancer; JAK1; STAT3

1. Introduction

Cervical cancer is one of the most common gynecological malignant tumors, and its incidence tends to be younger in recent years worldwide [1]. Radiotherapy, chemotherapy and surgery have been used in the treatment of cervical cancer, but the satisfactory results have not been achieved [2]. Radiotherapy can reduce surgical complications, but it causes side effects, including skin erythema, wet desquamation and mucositis. Besides, it also includes fibrosis, atrophy nerve damage and a series of growth and endocrine-related side effects [3–5]. In addition, drug resistance in the treatment of cervical cancer is a major obstacle for clinical treatment. Therefore, there is an urgent need to find an effective and reasonable treatment for cervical cancer.

SOCS4 is a negative regulator of cell signal transduction, which is abnormally expressed in tumor tissues and act as a tumor suppressor gene [6]. Studies have shown that targeted inhibition of SOCS4 by miR-1290 could activate the JAK1/STAT3 and PI3K/AKT pathways, promoting the growth, invasion and metastasis of lung adenocarcinoma cells [7]. Animal studies have found that the decreased SOCS4 expression level could enhance the activity of STAT3 before epithelial neoplasia [8]. Ma *et al.* [9] have showed that miR-944 affected the growth and invasion of non-small cell lung cancer by targeting SOCS4, indicating that SOCS4 plays a crucial role in non-small cell lung cancer. However, there are few studies considering the effects of SOCS4 on the growth, migration, invasion and apoptosis of cervical cancer cells and the relevant regulatory mechanisms are also little known.

Signal transducer and activator of transcription (STAT) families play a crucial role in the JAK-STAT pathway [10]. Among them, STAT3, as a member of the STAT family, is associated with malignant transformation of various tumors. It can promote tumor proliferation, invasion and metastasis while inhibit pro-inflammatory cytokines produced by tumor cells, which contributes to immune escape [11–13]. Studies have found that STAT3 overexpression is associated with cisplatin (cis-diamminedichloroplatinum (II), DDP) resistance in cervical cancer. STAT3 reduction significantly promoted CaSki cell apoptosis and reduced the resistance to DDP in cervical cancer [14]. Other studies have shown that SOCS4 could reduce STAT3 signal transduction [15]. Therefore, inhibiting STAT3 activity may have a potential anti-tumor effect. This investigation studied the effect of SOCS4 on the biological behavior of cervical cancer cells and explored the related underlying mechanisms.

2. Materials and methods

2.1 Cell culture

Four human cervical cancer cell lines (HeLa, SW-732, AV3, and CaSki) and the human normal cervical epithelium immortalized cell line (H8) were obtained from the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in culture medium (DMEM or RPMI-1640; Gibco, Carlsbad, CA, USA) with 10% FBS (Gibco), and 1% p/s (Gibco) in 5% CO_2 incubator.

2.2 qPCR

By using trizol reagent (Invitrogen, Carlsbad, CA, USA), the total RNA were obtained and its quantity and integrity were evaluated on a Nanodrop 1000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The PCR primers were designed and synthesized by Hanbio Technology (Shanghai, China). GAPDH was used as an endogenous reference gene to normalize mRNA expression levels. The relative expression level of SOCS4 in each experimental group was analyzed using the $2^{-\triangle\triangle Ct}$ method. The primers sequence in this paper was showed as follow Table 1.

Table 1. Primers for SOCS4 and reference genes.

Gene	Primer	Sequence(5'→3')
SOCS4	Forward	CGGGAGGAGTTCATCTGTT
	Reverse	GTTTCTTCTGGGCACTTTCT
GAPDH	Forward	CTGGGCTACACTGAGCACC
	Reverse	AAGTGGTCGTTGAGGGCAATG

2.3 Western blot

Briefly, cells were washed by pre-cooling PBS buffer for three times, and the total protein was separated by RIPA (Beyotime, Shanghai, China). An equal amount of total proteins was electrophoresed to SDS-PAGE. Then, they were transferred to the PVDF (Millipore) and treated by 5% non-fat milk for 1.5 h. The protein was incubated with specific primary antibodies including SOCS4 (Rabbit Anti-SOCS4 antibody, ab3607, 1 : 3000; Abcam, Cambridge, MA, USA), PCNA (Rabbit Anti-PCNA antibody, ab29, 1 : 3000; Abcam), MMP2 (Rabbit Anti-MMP2 antibody, ab92536, 1 : 3000; Abcam), MMP9 (Rabbit Anti-MMP9 antibody, ab76003, 1 : 3000; Abcam), Bax (Rabbit Anti-Bax antibody, ab32503, 1 : 3000; Abcam), Bcl-2 (Rabbit Anti-Bcl-2 antibody, ab182858, 1 : 3000; Abcam), JAK1 (Rabbit Anti-JAK1 antibody, ab133666, 1 : 3000; Abcam), p-JAK1 (Rabbit Anti-JAK1 (phospho Y1022 + Y1023) antibody, ab138005, 1 : 3000; Abcam), STAT3 (Rabbit Anti-STAT3 antibody, ab68153, 1 : 3000; Abcam), p-STAT3 (Rabbit Anti-STAT3 (phospho Y705) antibody, ab76315, 1 : 3000; Abcam), and GAPDH (Rabbit Anti-GAPDH antibody, ab9485, 1 : 2500; Abcam) overnight at 4 °C. Then, the membranes were further incubated with HRP-conjugated goat anti-rabbit immunoglobulin G secondary antibody (ab205718, 1 : 2000; Abcam) and the bands on the membranes were visualized by the ECL chemiluminescence reagent (Beyotime Biotechnology, Shanghai, China). The analyzed samples were normalized by GAPDH and the protein bands were quantified by gray value analysis by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.4 Cell transfection

To verify the effect of SOCS4 overexpression, vectors with or without SOCS4 sequences were transfected into HeLa cells. The synthetic of two kinds of vectors were from GenePharma (Shanghai, China). After cells were cultured for 24 hand transfected into HeLa cells by Lipofectamine 2000 (Invitrogen).

2.5 MTT assay

To assess the extent of cell proliferation, an MTT assay was applied. Briefly, 2.8×10^3 cells/mL cells were treated in different conditions as indicated in each experiment in triplicates. Following treatment, a final concentration of 0.5 mg/mL MTT solution (Beyotime) was added into each well, and the cells were cultured for another 4 h. Subsequently, 100 μL Dimethyl Sulfoxide (DMSO, Sigma) was added to visualize after discarding the culture medium. The optical density value of each samples was detected at 490 nm through a microplate reader (BioTek, Winooski, VT, USA).

2.6 Scratch assay

To assess the extent of cell migration, a scratch assay was used. Briefly, 4.8×10^5 cells were treated in different conditions as indicated in each experiment. The next day, mitomycin (1 μg/mL) was added to the plate for 1 h to inhibit cell division and a horizontal line on the surface of the plate was scratched by a pipette tip and a ruler. Following treatment, the plate was softly rinsed for 3 times by PBS and the cells were cultured in culture medium in 5% CO_2 incubator. The photos were taken 24 h after the incubation.

2.7 Transwell assay

The transwell assay was used to assess cell invasion. Briefly, 2.5×10^4 cells were plated into pre-coated 12-well transwell inserts (Costar, Manassas, VA, USA) in triplicates and treated under different conditions as indicated in each experiment. 220 μL of serum-free medium was added to the transwell inserts and 580 μL of medium with 10% FBS was added to the well. After culture for 24 h, cells that migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde for 15 minutes and dyed with 0.1% crystal violet solution for 25 minutes. The number of cells was counted finally.

2.8 Cell apoptosis

Annexin V/FITC and PI apoptosis detection kit (Invitrogen) and flow cytometry (BD Accuri^{TM} C6, Accuri Cytometers, Ann Arbor, MI, USA) were used to assesses cell apoptosis. Briefly, cells were digested and resuspended in incubation solution followed by staining with Annexin V/FITC and PI. The cells were cultured in the dark for 15 minutes and quantified using flow cytometry analysis.

2.9 Statistical analysis

All data were shown as mean ± standard error from 3 independent experiments. p values of <0.01 (two-tailed) were considered to indicate a statistically significant difference. GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) was used for analysis.

3. Results

3.1 SOCS4 showed low expression in human cervical cancer cell lines

In order to investigate the expression of SOCS4 in cervical cancer, the mRNA and protein expression of SOCS4 in human normal cervical epithelium immortalized cell line (H8) and four human cervical cancer cell line (HeLa, SW-732, AV3, and CaSki) were determined by qPCR and western blot, respectively. The results revealed that the mRNA and protein expression of SOCS4 was obviously decreased in all kinds of cervical cancer cell lines compared to H8 cell line (Fig. 1A,B). Then, in order to stably overexpress SOCS4 *in vivo*, the constructed vectors were transfected into HeLa cells and its mRNA and protein expression level was also confirmed by qPCR and western blot. The results confirmed that the mRNA and protein expression levels of SOCS4 in SOCS4 overexpression group (SOCS4) were significantly higher than that in the control group (Control) or NC vector group (Vector), indicating that SOCS4 was stably overexpressed in HeLa cells (Fig. 1C,D). These results verified that SOCS4 was lowly expressed in human cervical cancer cell lines and its mRNA and protein expression level could be stably overexpressed in HeLa cells by cell transfection.

3.2 Overexpression of SOCS4 inhibited the proliferation, migration and invasion of cervical cancer cells

MTT assay was carried out to detect the effect of SOCS4 on the cervical cancer cell growth. The results showed that, compared with the control group (Control) or NC vectors group (Vector), overexpression of SOCS4 markedly inhibited the proliferative ability of HeLa cells (Fig. 2A). In addition, the wound scratch healing assay was carried out to detect the effect of SOCS4 on the cervical cancer cell migration. The results suggested that the mobility of HeLa cells was notably reduced by SOCS4 overexpression (Fig. 2B). Furthermore, the transwell assay was carried out to detect the effect of SOCS4 on cervical cancer cell invasion. The results indicated that the number of invaded HeLa cells was obviously decreased by SOCS4 overexpression (Fig. 2C). In order to further clearly investigate whether some other factors involved in the growth, migration and invasion of cervical cancer cells were affected by SOCS4 overexpression, the expression levels of tumor cell proliferation factor (PCNA) and cell migration related proteins (MMP4 and MMP9) were detected by western blot. The results confirmed that the expression levels of tumor cell proliferation factor or cell migration related proteins in HeLa cells were dramatically lower by SOCS4 overexpression (Fig. 2D). These results confirmed that overexpression of SOCS4 inhibited the proliferation, migration and invasion of cervical cancer cells.

3.3 Overexpression of SOCS4 promoted apoptosis of cervical cancer cells

To explore the role of SOCS4 in the apoptosis of cervical cancer cells, flow cytometry assays were performed. The flow cytometry assay confirmed that overexpression of SOCS4 promoted the apoptosis of cervical cancer cells, as evidenced by increased apoptotic cell number after SOCS4 overexpression transfection than control group (Control) or NC vector group (Vector) (Fig. 3A). In addition, the levels of cell apoptosis-related proteins were also examined by western blot, and the results confirmed that overexpression of SOCS4 dramatically increased the expression of Bax while strikingly decreased the expression level of Bcl-2 (Fig. 3B). These results suggested that the apoptosis of cervical cancer cells could be enlarged by upregulating the level of SOCS4.

3.4 Overexpression of SOCS4 inhibited the JAK1/STAT3 pathway activation

To explore whether the mechanism underlying the effects of SOCS4 overexpression on promoting the growth and migration of cervical cancer cells was mediated by JAK1/STAT3 pathway, western blot essay was used to detect the expression of JAK1 and STAT3 in HeLa cells. The phosphorylation levels of JAK1 and STAT3 proteins showed a dramatic downregulation in HeLa cells after SOCS4 was overexpressed, indicating that SOCS4 could inactivate JAK1 and STAT3 signaling pathway (Fig. 4A,B). These data indicated that SOCS4 was involved in the inactivation of JAK1/STAT3signaling pathway.

4. Discussion

Recently, some studies have suggested that SOCS4 has the potential to plays a crucial role in tumor development [16]. However, SOCS4 is rarely studied in cervical cancer, and understanding the molecular regulatory mechanism of SOCS4 may provide the novel therapeutic targets for the treatment of cervical cancer. Therefore, this study is focus on the molecular regulatory mechanism of SOCS4 in cervical cancer fate determination.

Suppressors of cytokine signaling (SOCS) proteins has been shown to play vital roles in modulating cell growth and migration processes [17]. Many SOCS proteins are often expressed in tissues or organs, contributing to the maintenance of normal tissue functions and homeostasis, which thus affect cell growth and migration [18]. Moreover, many tripartite motif containing proteins are implicated in cancer-associated cell growth and migration, and disorders of tripartite motif containing proteins play a vital role in the occurrence and progression of cancer [15]. In particular, studies have found that miR-106a-3p induces apatinib resistance in gastric cancer via activating the JAK2/STAT3 signaling pathway by targeting SOCS system [19]. Recently, the relationship between miR-101 level and SOCS-2 expression in the inhibition of ovarian cancer cell proliferation and invasion was confirmed [20]. Moreover, SOCS-1 was found to ex-

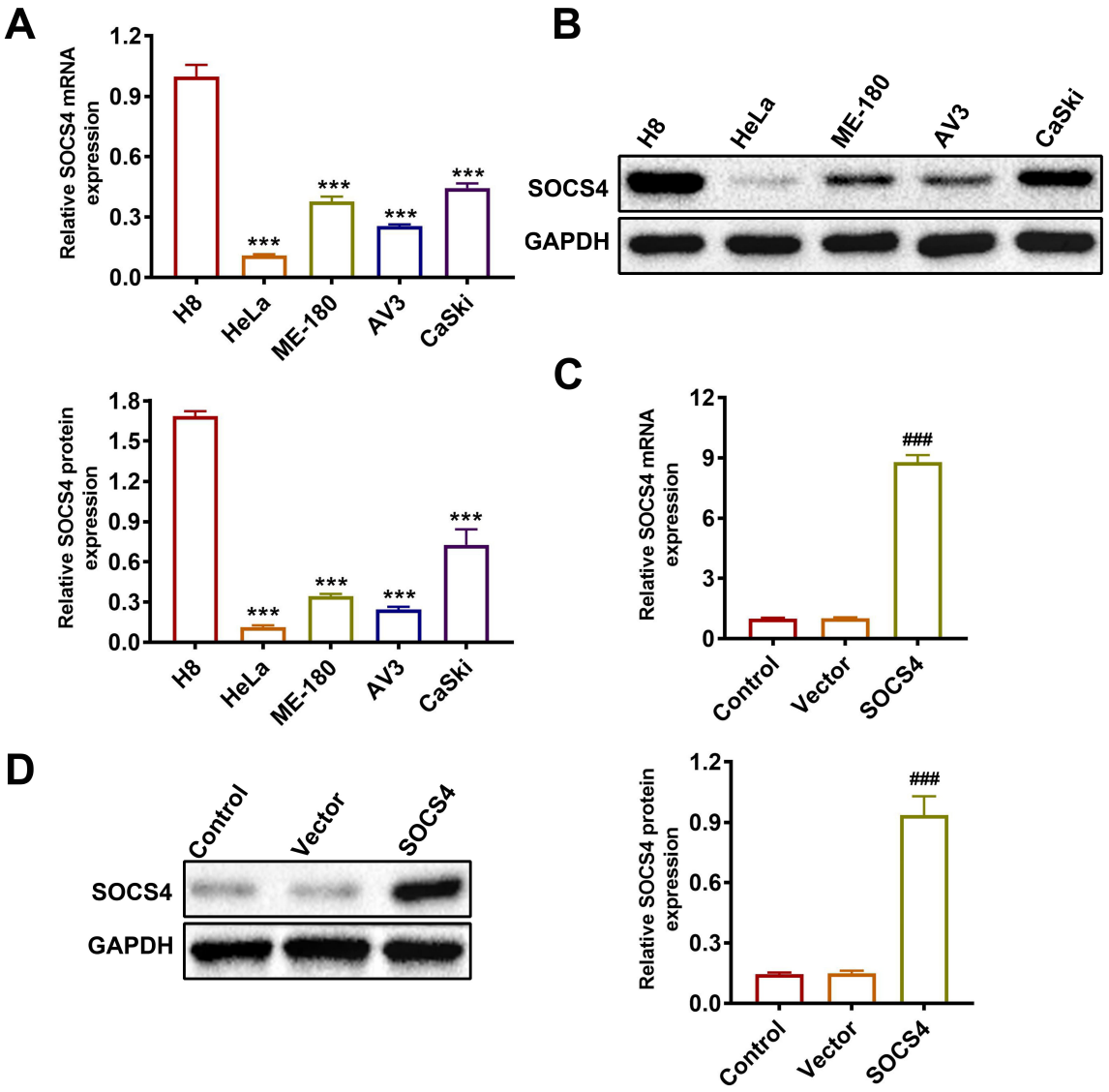

Fig. 1. SOCS4 showed low expression in human cervical cancer cell lines. (A) The mRNA expression level of SOCS4 in human normal cervical epithelium immortalized cell line (H8) and four human cervical cancer cell lines (HeLa, SW-732, AV3, and CaSki). (B) The protein expression level of SOCS4 in human normal cervical epithelium immortalized cell line (H8) and four human cervical cancer cell lines (HeLa, SW-732, AV3, and CaSki). (C) Overexpression of SOCS4 in HeLa was verified by qRT-PCR. (D) Overexpression of SOCS4 in HeLa was verified by western blot. Data were presented as the mean ± SD with three independent experiments. $^{***}p < 0.001$; $^{###}p < 0.001$.

ert anti-proliferative effect in gastric cancer cells via STAT3 and MAPK signaling pathway [21]. This study disclosed that the mRNA and protein expression of SOCS4 was lower in four human cervical cancer cell lines (HeLa, SW-732, AV3, and CaSki) than normal cervical epithelium immortalized cell line (H8). Besides, overexpression of SOCS4 inhibited the proliferation, migration, and invasion of cervical cancer cells. These results confirmed that SOCS4 may act as an inhibitory factor in the growth and migration of cervical cancer cells.

A growing number of researches confirmed that SOCS proteins exert their functions by regulating the expression of target mRNAs [22, 23]. A previous study proved that SOCS5 regulates autophagy to impair metastasis via PI3K/Akt/mTOR pathway in hepatocellular carcinoma cells [24]. Moreover, SOCS3 was reported to antagonise the proliferative and migratory effects by inhibition of p44/p42 MAPK pathway in prostate cancer [25]. In addition, hypermethylation of SOCS1 was reported to mediate activation of IL-6 via JAK/STAT pathway in human gastric cancer [26]. Another novel discovery of this study was JAK1 and STAT3 were directly targeted by SOCS4. JAK1 is a human tyrosine kinase protein that is essential for signal transduction of certain cytokines. STAT3 is a member of the STAT protein family, which plays a pivotal role in multiple cellular processes including cell proliferation and migration. Here, the fact that SOCS4 could target JAK1 and STAT3 was demonstrated by western blot assays. Overexpression of SOCS4 in HeLa cells could inhibit the phosphorylation of JAK1 and

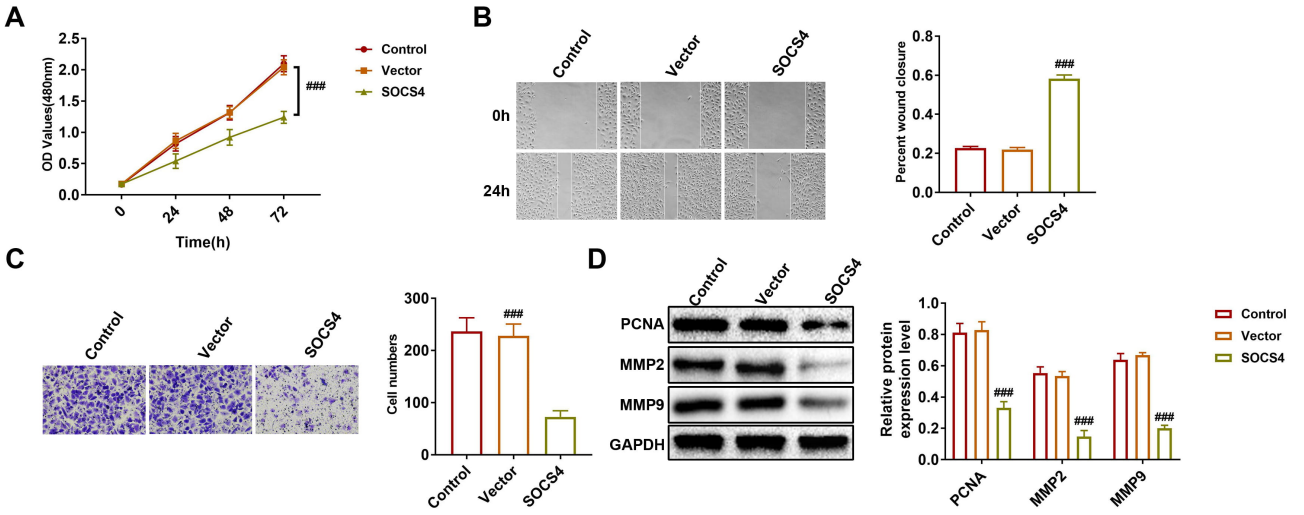

Fig. 2. Overexpression of SOCS4 inhibited the proliferation, migration and invasion of cervical cancer cells. (A) Serial MTT cell viability assays detected the proliferation of HeLa cells after overexpression of SOCS4. (B) Wound scratch healing assay of HeLa cells after overexpression of SOCS4. (C) Transwell assay of HeLa cells after overexpression of SOCS4. (D) The expression levels of tumor cell proliferation factor PCNA and cell migration related proteins MMP4 and MMP9 were detected by western blot. Data were presented as the mean ± SD with three independent experiments. $^{###}p < 0.001$.

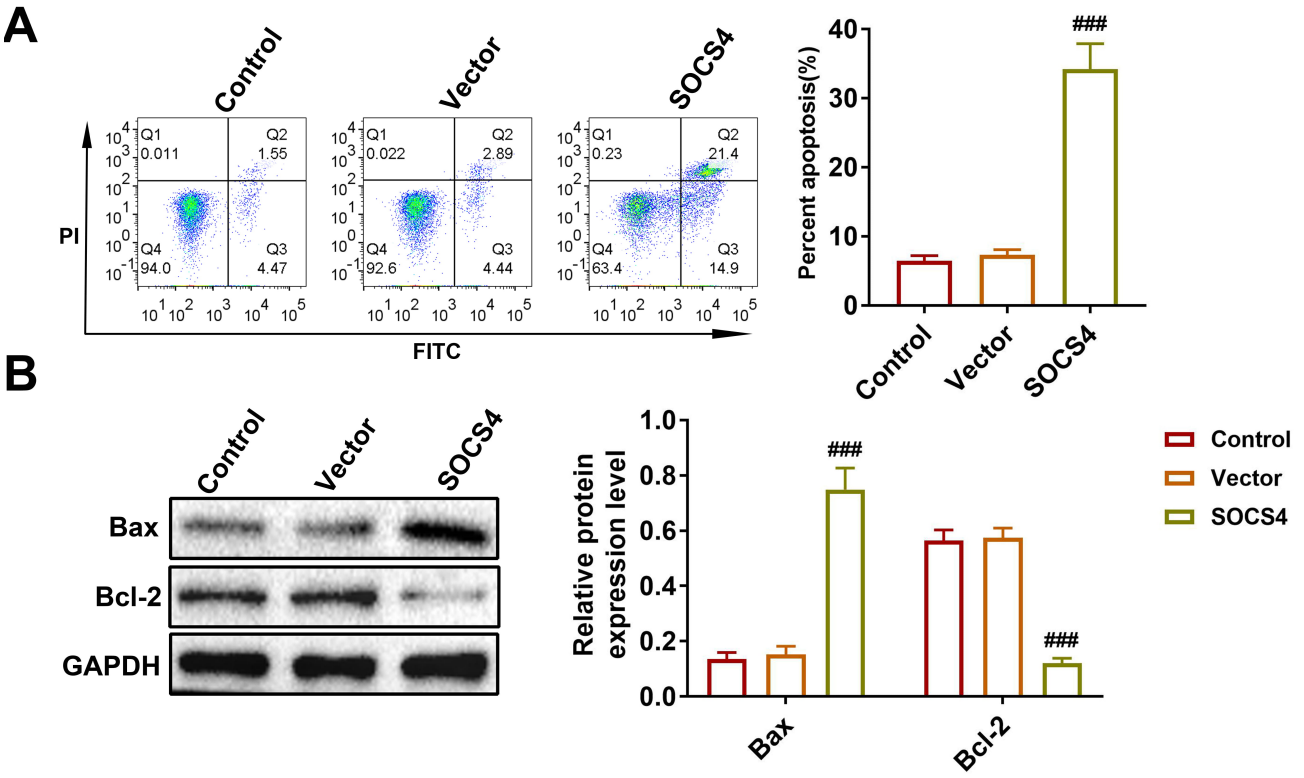

Fig. 3. Overexpression of SOCS4 promoted apoptosis of cervical cancer cells. (A) Flow cytometry analysis was applied to detect apoptosis in vectors group (SOCS4), control group (Control), and NC vectors group (Vector). (B) Western blot was applied to detect cell apoptosis-related proteins. Data were presented as the mean ± SD with three independent experiments. $^{###}p < 0.001$.

STAT3 protein, indicating that SOCS4 inhibited the proliferation and migration of cervical cancer cells by regulating the JAK1/STAT3 pathway.

In conclusion, in this study, the fact that SOCS4 was downregulated in human cervical cancer cell lines was discovered. Moreover, overexpression of SOCS4 inhibited the proliferation, migration and invasion of cervical cancer cells. Meanwhile, overexpression of SOCS4 promotes the apoptosis of cervical cancer cells. Finally, overexpression of SOCS4 in HeLa cells could inhibit the phosphorylation of JAK1 and STAT3 protein. These results figured out the role of SOCS4/JAK1/STAT3 signaling pathway in inhibiting the

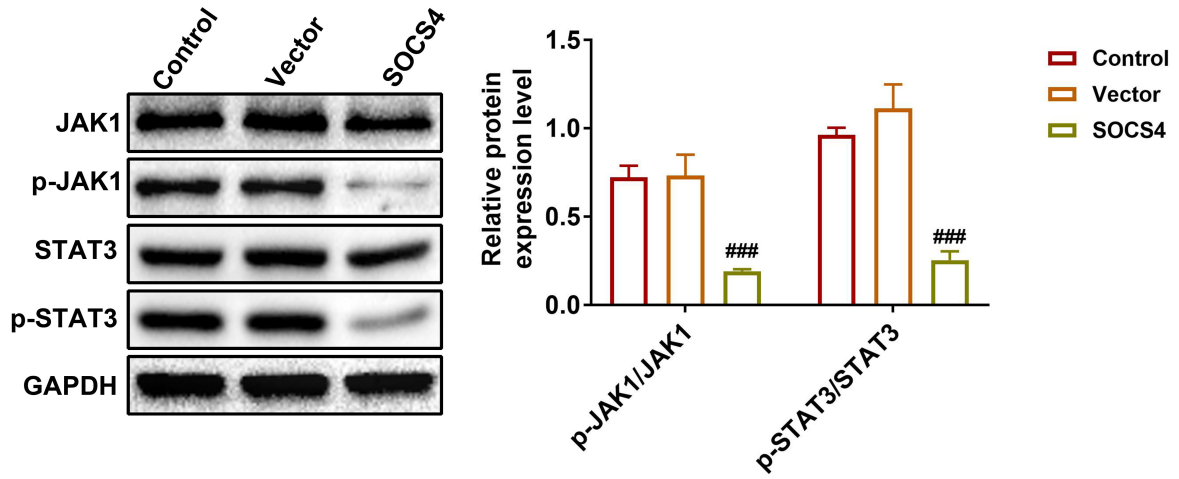

Fig. 4. Overexpression of SOCS4 inhibited the JAK1/STAT3 signaling pathway activation. The protein levels of JAK1, p-JAK1, STAT3, and p-STAT3 in HeLa cells after overexpression of SOCS4, as determined using western blotting. Relatively quantitative results were determined by Image J and shown as histogram. Data were presented as the mean ± SD with three independent experiments. $^{###}p < 0.001$.

proliferation and migration of cervical cancer cells, which could conceivably pave the path for advanced therapeutic targets for the treatment of cervical cancer.

Author contributions

SHC and YHC designed the study, supervised the data collection, LTY analyzed the data, interpreted the data, XMH prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki, and the 264 protocol was approved by the Ethics Committee of Changsha Maternal and Child Health Care Hospital (approval number: 2019-052).

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Conflict of interest

The authors declare no conflict of interest.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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