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MiR-203 regulates proliferation and apoptosis of ovarian cancer cells by targeting SOCS3

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Abstract. – OBJECTIVE: Cytokine signal transduction inhibitor 3 (SOCS3) negatively regulates Janus kinases (JAK) - signal transducer and activator of transcription (STAT) pathway. Bioinformatics analysis revealed a targeted relationship between miR-203 and SOCS3 mRNA. This study investigated the role of miR-203 in ovarian cancer cell proliferation and apoptosis.

PATIENTS AND METHODS: Ovarian cancer tissues and adjacent tissues were collected to detect the expression of miR-203 and SOCS3. Ovarian cancer HO8910 cells were divided into miR-NC group, miR-203 inhibitor grou[illegible] miR-203 mimic group followed by the a[illegible] of the expression of miR-203 and SOCS[illegible]R-NA by quantitative Reverse Transcription-[illegible] (qRT-PCR), protein expression of p-JAK2 [illegible] p-STAT3 by Western blot, cell apoptosis by flo[illegible] cytometry, and proliferation b[illegible]nyl-2'-de[illegible] oxyuridine (EdU) staining ch[illegible]olog[illegible]y.

RESULTS: Compared w[illegible] adjace[illegible] tissues, miR-203 expression was [illegible]ificantl[illegible]ed in tumor tissues and SC[illegible]RN[illegible] was decreased. Com[illegible]ed w[illegible]e with lower miR-203 expressio[illegible]he prog[illegible]of patients with higher exp[illegible]n of miR-20[illegible] signifi-cantly worse. [illegible] a targeted [illegible]gulatory relationship [illegible]ween [illegible]203 and SOCS3 mR-NA. Comp[illegible]ed with IO[illegible]cells, miR-203 ex-pression [illegible]HO8910 and [illegible]3 cells was in-crease[illegible]d its expressions [illegible] SOCS3 mRNA and [illegible]ein w[illegible]decreased. Compared with mi[illegible] grou[illegible]e transfection of miR-203 in-hibi[illegible]ntly in[illegible]ased SOCS3 expres-sion, a[illegible]reased [illegible] expression of p-JAK2 [illegible]-ST[illegible]rote[illegible] We draw the conclusion [illegible]iR-20[illegible]ed cell apoptosis and de-crea[illegible]d cell p[illegible]eration. However, opposite res[illegible]were observed after the transfection of m[illegible]

CONCLUSIONS: Abnormal miR-203 and SOCS3 [illegible]pression are related to the pathogenesis of [illegible]n cancer. MiR-203 affects the proliferation of JA[illegible]AT pathway and regulates the proliferation and apoptosis of ovarian cancer cells by targeting the inhibition of SOCS3 expression.

Key Words:
MiR-203[illegible]K-STAT, Ovaria[illegible]ancer.

Introduction

[illegible]varian canc[illegible]OC) is one of the most com-m[illegible]alignant [illegible]ors of the female reproductive syst[illegible]been the 5th leading cause of death i[illegible]s of female malignant tumor-relat-[illegible]d mortality, which poses a serious threat to the [illegible]d quality of life of female population[1].

[illegible]AK-STAT signaling pathway is involved in various biological processes, such as cell survival, proliferation, migration, and invasion[2,3]. SOCS3 is one of the most important and widely expressed member of the cytokine signaling (SOCS) family, which directly inhibits the activity of JAK kinase and phosphorylation of its downstream STAT protein, blocking the activation of the JAK-STAT signaling pathway[4]. Studies[5-7] have shown that, as a tumor suppressor gene, abnormal expression or functioning of SOCS3 is associated with the occurrence, progression, metastasis, and prognosis of ovarian cancer.

MicroRNA is an endogenous non-coding small-molecule single-stranded RNA of eukaryotes with a length of about 22-25 nucleotides, which binds to the 3'-Untranslated Region (UTR) of the target gene mRNA by complementary pairing to degrade or inhibit translation. MicroRNAs regulate the expression of target genes, which account for 1% of human genes[8-10]. In ovarian cancer-related studies, several researches[11-14] have shown that the abnormal expression of miR-203 is closely related to the occurrence, progression, metastasis, and prognosis of ovarian cancer, suggesting that miR-203 plays the role of both tumor

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suppressor and cancer-promoting gene in ovarian cancer. Bioinformatics analysis revealed a targeted binding site located between miR-203 and 3'-UTR of SOCS3 mRNA. This study investigated the role that miR-203 plays in terms of regulating SOCS3 expression and affecting JAK2-STAT3 pathway activity in ovarian cancer cell proliferation and apoptosis.

Patients and Methods

Main Reagents and Materials

Human normal ovarian epithelial cells IOSE80, ovarian cancer HO8910, SKOV3 cells were purchased from Sino Biological (Beijing, China); HEK293T cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China); Dulbecco's Modified Eagle's Medium (DMEM) and Opti-MEM were purchased from Gibco (Grand Island, NY, USA); fetal bovine serum (FBS) was purchased from Yikesai Biotechnology (Shanghai, China); Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA); PrimeScript™ RT reagent Kit was purchased from TaKaRa (Dalian, China); SYBR Green dye purchased from Toyobo (Osaka, Japan); miR-203 inhibitor, miR-NC, and miR-203 mimic were designed and synthesized by Ribobo Bio (Guangzhou, China), Rabbit anti-human SOCS3, p-JAK2, p-STAT3 antibody were purchased from Abcam (Cambridge, MA, USA); rabbit anti-human actin polyclonal antibody was purchased from [illegible] China); Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Jackson Immunoresearch (West Grove, PA, USA); pGL3 vector was purchased by BioVector (Beijing, China); Dual-Luciferase activity assay kit purchased from Promega (Madison, WI, USA); Annexin V-Fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) apoptosis assay reagent purchased from Keluo Lab bio (Beijing, China); EdU-Alexa Fluor 488 cell proliferation assay kit purchased from Thermo Fisher Scientific (Waltham, MA, USA), and commonly used related reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Clinical Data

92 patients with ovarian cancer who were treated in The 960th Hospital of the PLA Joint Logistics Support Force from January 2016 to March 2019 were selected, with an average age of 43.6±11.8 years. The tumor tissues that have been removed during the operation and the specimens of the adjacent tissues more than 2 cm away from the tumor tissues were collected, and the tissue specimens were confirmed by pathological examination. Patients were informed about the collections of the tissues and the procedure was reviewed and approved by The 960th Hospital of the PLA Joint Logistics Support Force Ethics Committee.

Cell Culture

IOSE80, HO8910 and SKOV3 cells were inoculated in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% FBS and 1% penicillin and cultured in a cell culture incubator containing CO_2 at 37°C. When the process was completed, cells were collected by enzymatic digestion, subcultured at a ratio of 1:3 to 1:6. The investigation was performed when cells were in logarithmic growth phase.

Dual-Luciferase Reporter Gene Test

The length of the 3'-UTR fragment of the SOCS3 gene or the fragment containing the mutant was amplified using the HEK293T genome as a template, and was cloned into the pGL3 vector, transformed into DH5α competent cells, and the correct plasmids were sequenced as 5' AUUCAGAAAAGAAACAUUUCA 3', 3' GAUCACCAGGAUUUGUAAAGU 5' and 5' AUUCAGAAAAGCAGCGCAUGA 3' and named as pGL3-SOCS3-WT, pGL3-SOCS3-MUT. pGL3-SOCS3-WT (or pMIR-SOCS3-MUT) and miR-203 mimic (or miR-NC) were co-transfected into HEK293T cells with Lipofectamine 2000 reagent. After 48 hours of cells culture, the relative Luciferase activity was measured using the Dual-Luciferase Reporter Assay kit.

Cell Transfection

HO8910 cells were cultured *in vitro* and divided into 3 transfection groups, i.e., miR-NC transfection group, miR-203 inhibitor transfection group, and miR-203 mimic transfection group. The general procedure for transfection was as follows: 10 μL of Lipofectamine 2000 and 50 nmoL miR-NC (or miR-203 inhibitor or miR-203 mimic) were diluted with 100 μL of serum-free Opti-MEM, respectively, and then incubated for 5 min at room temperature, respectively, gently mixed and next, incubated for 20 min at room temperature. We added the transfectants to the

cell culture medium, mixed gently, continued to culture for other 72 hours, followed by detection of related indicators, such as protein and apoptosis.

QRT-PCR Detection of Gene Expression

Ribonucleic Acid (RNA) was extracted by TRIzol method, and was reversely transcribed to cDNA using PrimeScript™ RT reagent Kit, and the resulting cDNA was stored in a refrigerator at –20°C. Polymerase chain reaction (PCR) amplification was carried out under the action of Taq DNA polymerase using cDNA as a template in a total of 10 μL PCR reaction system (including 2×SYBR Green Mixture 5.0 μL, 2.5 μm/L forward primer 0.5 μL, 2.5 μm/L reverse primer 0.5 μL, cDNA 1 μL, and ddH2O). PCR conditions: 95°C 5 min, 95°C 15 s, 60°C 1 min, fluorescence data were stored on a Bio-Rad CFX96 real-time PCR instrument (manufacturer: Bio-Rad; model: CFX96, Hercules, CA, USA) for 40 cycles.

Western Blot

The cells were collected, and the total protein was extracted from the radioimmunoprecipi[tation] assay (RIPA) lysate. After the concentrat[ion] determined, 40 μg was applied to the s[ample], and electrophoresis was carried out for 3 h in sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel (8% separation gel, 5% concentrated gel). The protein was electroporated (300 mA), and then, transferred to polyvinylidene difluoride (PVDF) membrane for 90 min, followed by blocking with 5% skim powder in phosphate-buffered saline and Tween 20 (PBST) for 60 min at room temperature, and incubated with primary antibody at 4°C overnight (the dilution ratio of SOCS3, p-JAK2, p-STAT3, and β-actin was 1:2500, 1:1000, 1:1000, 1:8000, respectively), followed by washing 3 times with PBST, and incubated with HRP-labeled secondary antibody (1:5000 dilution) for 60 min at room temperature. After being washed 3 times with PBST, enhanced chemiluminescence (ECL) working solution was added to the blotting membrane and incubated for 1-3 min at room temperature, followed by the process in terms of exposure and development.

Flow Detection of Cell Apoptosis

The above-mentioned miR-NC, miR-203 inhibitor and miR-203 mimic transfection group HO8910 cells were collected by trypsinization. After being washed twice with PBS, 300 μL of Binding Buffer was added to the cell pellet and the cells were suspended, and 5 μL of Annexin V-FITC was added and incubated for 15 min in the dark. Then, 5 μL of PI staining was added, followed by the analysis of cell apoptosis by flow cytometry.

EdU Staining for Cell Proliferation

HO8910 cells were resuspended in 96-well plate with complete medium containing 10% FBS, incubated with 10 μM 5-Ethynyl-2'-deoxyuridine (EdU) for 120 min, transfected with miRNAs according to the procedure described above, and then, cultured for 72 hours. After transfection, the cells were collected according to the procedure. Based on the instructions, after the centrifugation, washing, fixation, permeabilization, and incubation of the reaction solution, cell proliferation was detected by Beckman Coulter FC 500 MCL flow cytometry (Brea, CA, USA), and the proliferation ability of the cells was reflected by the positive rate of EdU.

Statistical Analysis

Statistical analysis of the data was performed using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) 18.0 software. The measurement data were expressed as mean ± standard deviation (SD). The Student's *t*-test was used to compare the measurement data between the two groups. $p < 0.05$ was considered statistically significant.

Results

Abnormal Expression of MiR-203 and SOCS3 in Ovarian Cancer

The results of qRT-PCR showed that the expression of miR-203 was significantly increased in tumor tissues of ovarian cancer patients compared with adjacent tissues (Figure 1A). The results of qRT-PCR showed that the expression of SOCS3 mRNA in tumor tissues of ovarian cancer patients was significantly lower than that of adjacent tissues (Figure 1B).

High Expression of MiR-203 is Associated With Poor Prognosis in Z With Ovarian Cancer

The ovarian cancer patients were divided into miR-203 high expression group and miR-203 low expression group based on the median level of miR-203 mRNA expression. The relationship be-

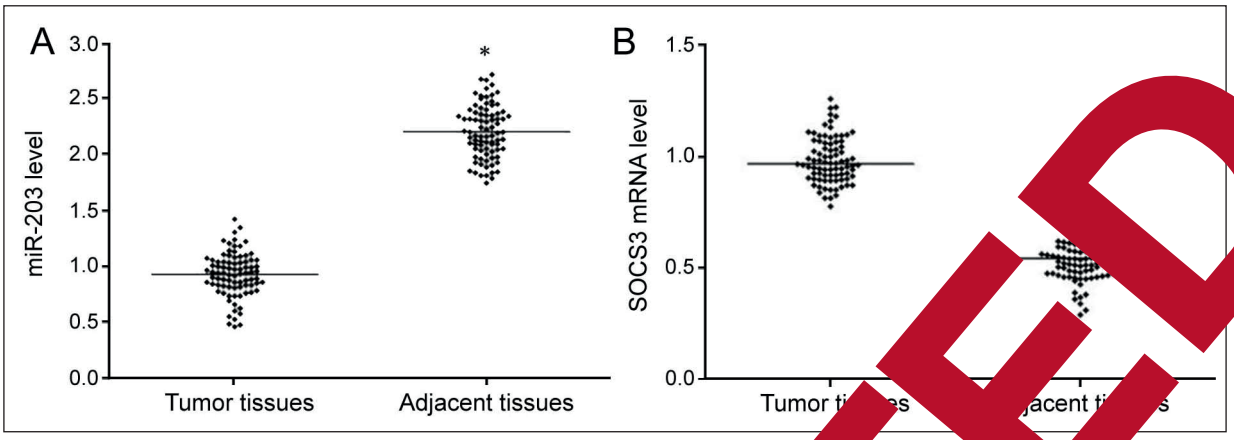
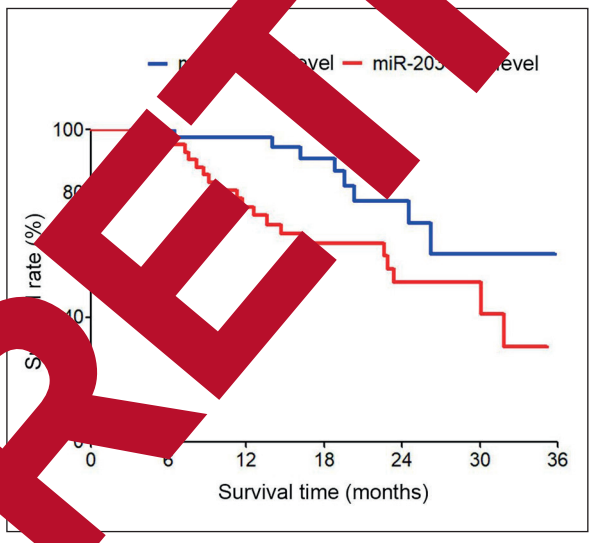


Figure 1. Abnormal expression of miR-203 and SOCS3 in ovarian cancer. **A,** Q R detection o xpression in ovarian cancer tissues. **B,** QRT-PCR detection of SOCS3 mRNA expression i ncer tissues. sents $p < 0.05$ compared with adjacent tissues.

tween miR-203 expression and survival rate and prognosis was analyzed. Survival curve analysis showed that the survival rate and prognosis of patients with high miR-203 expression were much worse than those with low expression of miR-203 (Log-lank test $\chi^2 = 4.506$, $p = 0.034$; Figure 2).

Targeted Regulation Relationship Between MiR-203 and SOCS3 mRN

Bioinformatics analysis revealed a comple tary binding site located between miR-203 and 3'-UTR of SOCS3 mRNA (Figu). Dual-L ciferase gene reporter assays t trans- fection of miR-203 mimic arkat reduced relative Luciferase activit pGL3-



Figur 2. High expression of miR-203 is associated with poor prognosis in patients with ovarian cancer.

tra HEK293T cel ut miR-NC or miR- mimic did not effect relative Luciferase ac- y in HEK29 cells transfected with pGL3- S3-MUT (F e 3B), indicating that miR- a target e regulatory relationship with the of SOCS3 mRNA.

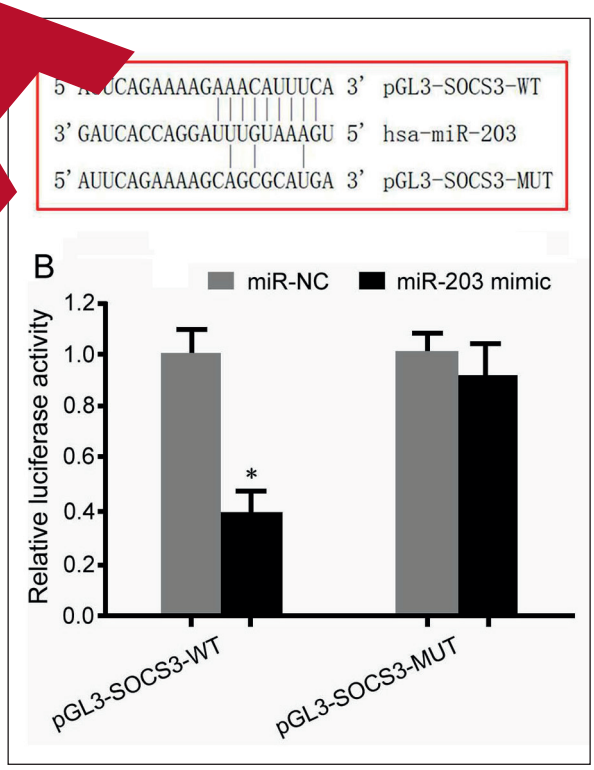


Figure 3. Targeted regulation relationship between miR-203 and SOCS3 mRNA. **A,** Schematic diagram of the interaction site between miR-203 and the 3'-UTR of SOCS3 mRNA. **B,** Dual-Luciferase gene reporter assay. *Represents $p < 0.05$ compared to miR-NC.

Increased Expression of miR-203 in Ovarian Cancer Cells and Decreased Expression of SOCS3

The results of qRT-PCR showed that compared with normal ovarian epithelial IOSE80 cells, the expression of miR-203 in ovarian cancer HO8910 and SKOV3 cells was significantly increased, while the expression of SOCS3 mRNA was significantly decreased (Figure 4A). Western blot analysis showed that the expression of SOCS3 protein in ovarian cancer HO8910 and SKOV3 cells was significantly lower than that in normal ovarian epithelial IOSE80 cells (Figure 4B).

Inhibition of miR-203 Expression Attenuates Ovarian Cancer Cell Proliferation and Induces Apoptosis

The results of qRT-PCR showed that compared with miR-NC transfection group, the expression of miR-203 was significantly decreased in HO8910 cells transfected with miR-203 inhibitor, whereas the expression of SOCS3 mRNA was significantly increased. The expression level of miR-203 was significantly increased in the HO8910 cells transfected with miR-203 m[illegible] while the expression level of SOCS3 mR[illegible] significantly decreased (Figure 5A). Weste[illegible]ot analysis showed that compared with mi[illegible] transfection group, transfection of miR-203 [illegible]hibitor significantly increased t[illegible]pression [illegible] SOCS3 protein in HO8910 cel[illegible]ificantly decreased the expression of [illegible]K2 ar[illegible]STAT3 protein. Transfection of [illegible]203 mi[illegible]used the expression of SOCS3 [illegible]in [illegible] the expression of p-JAK2 and p-STAT3 protein (Figure 5B). Flow cytometry results showed that the proliferation of HO8910 cells in the [illegible] inhibitor transfection group was sign[illegible] duced compared with miR-NC tran[illegible]tion group (Figure 5C), and cell apoptosis [illegible]ignificantly increased (Figure 5D) On the oth[illegible]d, miR-203 mimic transfection si[illegible]ificantly [illegible]ased the proliferation of HO89[illegible]ls and red[illegible] apoptosis.

Discu[illegible]

Ovarian c[illegible] is rather ins[illegible]n the early stage. Yet [illegible]w[illegible]own for its [illegible]apid progression. Most [illegible]atients [illegible] the middle or late stages of the di[illegible]se when th[illegible] admitted to hospital fo[illegible]nt, which some[illegible]at added difficulties t[illegible]e treatment[15,16]. Therefore, to explore the [illegible]ogenesis of [illegible]rian cancer and to find the [illegible]l molecules [illegible] abnormal changes have the p[illegible]ial of gre[illegible]gnificance in helping to diagnos[illegible] therapeutic effect, as well as the prog[illegible]of patients.

When a cytokine, i.e., a growth factor or a [illegible]binds to an intracellular receptor, the [illegible]cep[illegible] can form a homologous or heterodimer, and phosphorylate the JAK kinase. The activated JAK can make the receptor phosphorylation of the tyrosine residue of STAT to complement STAT via the SH2 domain to the tyrosine phosphorylation site of the receptor complex, at which point JAK kinase is spatially adjacent

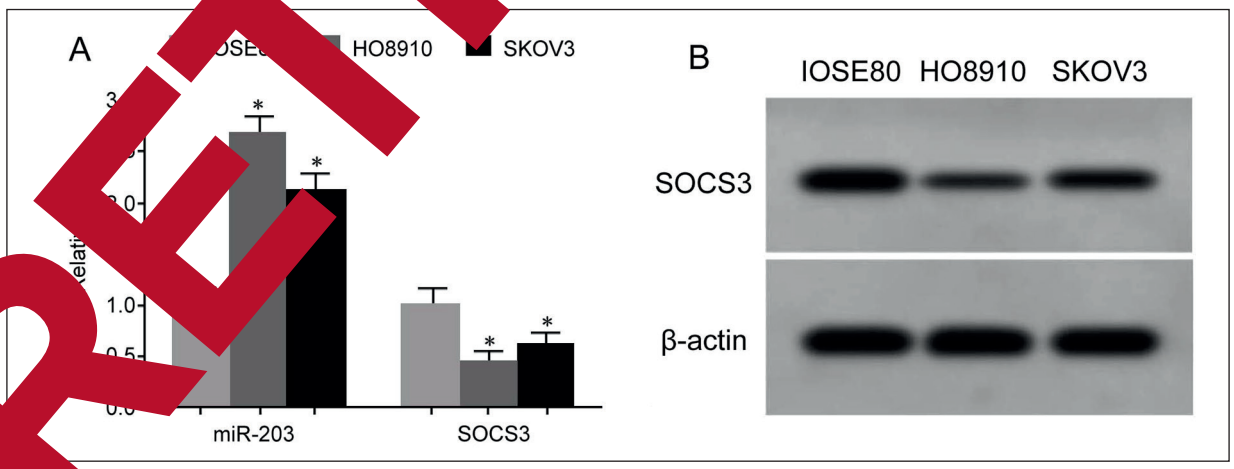


Figure 4. Increased expression of miR-203 in pancreatic cancer cells and decreased expression of SOCS3. **A,** QRT-PCR was used to detect the expression of miR-203 and SOCS3 mRNA in cells. **B,** Western blot was used to detect the expression of SOCS3 protein in cells. *Represents $p < 0.05$ compared to IOSE80 cells.

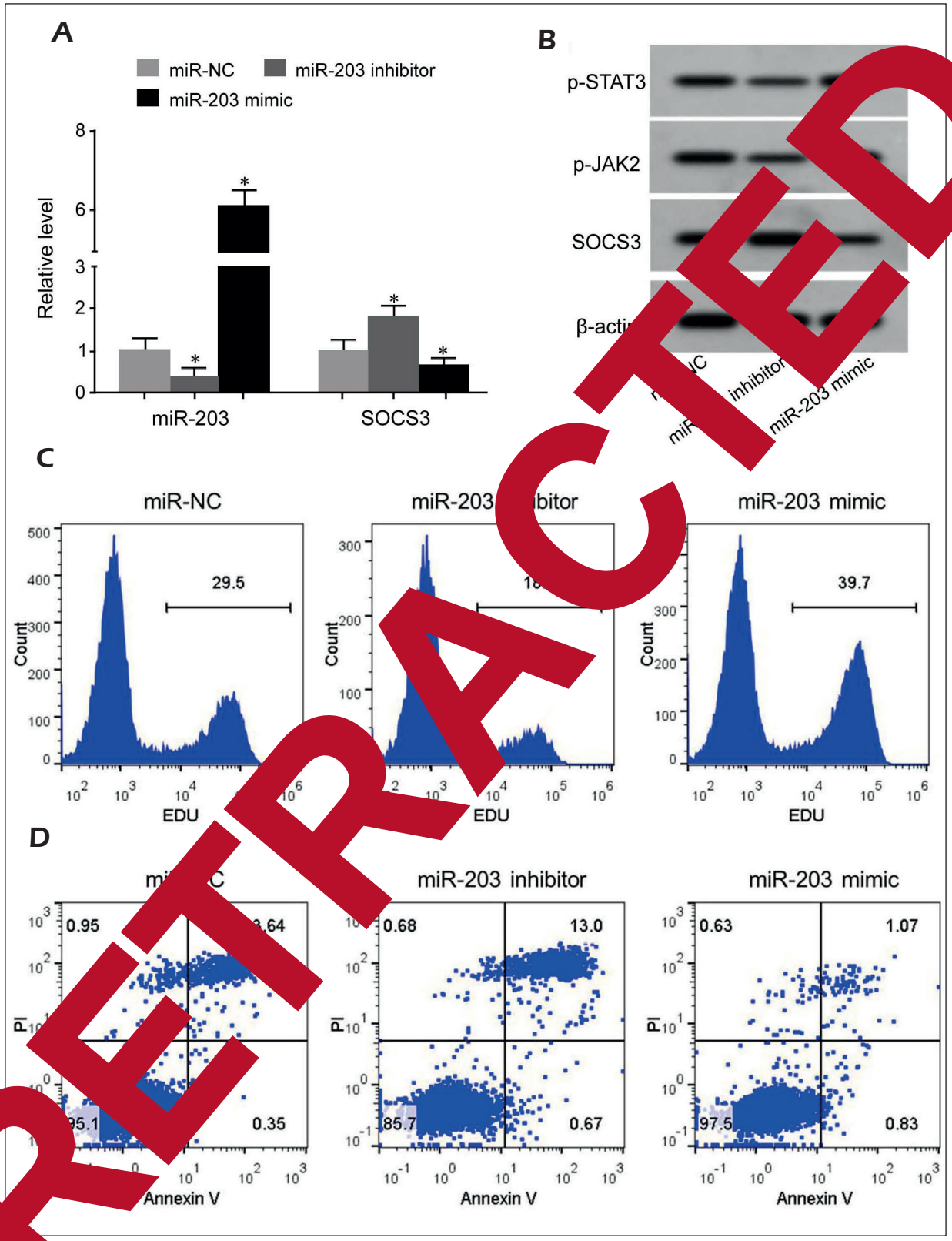


Fig. 3. Inhibition of miR-203 expression attenuates ovarian cancer cell proliferation and induces apoptosis. **A,** QRT-PCR detection of gene expression. **B,** Western blot detection of protein expression. **C,** Flow detection of cell proliferation. **D,** Flow detection of apoptosis. *Represents $p < 0.05$ compared to miR-NC.

to STAT and phosphorylates its hydroxytyrosine site, phosphorylated and activated STAT is separated from the receptor complex, forms a dimer and transports from the cytoplasm to the nucleus, acts on specific DNA fragments, and regulates gene transcription and expression[2,17,18]. SOCS3 is one of the most important and widely expressed members of the SOCS family. SOCS3 directly inhibits JAK kinase activity and STAT phosphorylation, and inhibits the activity and transmission of JAK-STAT signaling pathway[4,6]. As an important tumor suppressor, the expression or decreased function of SOCS3 facilitates the occurrence, progression, and metastasis of various tumors[19-21]. Authors[5-7] have found that, as a tumor suppressor gene, abnormal expression or functioning of SOCS3 is associated with the occurrence, progression, metastasis, and prognosis of ovarian cancer.

Several researches have indicated that the expression and function of miR-203 are abnormally altered in gastric cancer[22], intestinal cancer[23], breast cancer[11], and other tumors. This study investigated the role miR-203 plays in terms of regulating SOCS3 expression and aff[illegible] JAK2-STAT3 pathway activity in ovarian cell proliferation and apoptosis.

The results of qRT-PCR showed that comp[illegible] with adjacent tissues, the expression of miR-[illegible] was significantly increased in t[illegible] tissues [illegible] ovarian cancer patients, wh[illegible] pression of SOCS3 mRNA was si[illegible]cantly [illegible]reased, suggesting that the expr[illegible]n of mi[illegible] plays a role in reducing the exp[illegible]n [illegible] promoting the path[illegible]esis [illegible]ian cancer. Survival analysis [illegible]ved that t[illegible]rvival rate and prognosis [illegible]nts with hi[illegible] expression of miR-2[illegible] we[illegible]nificantly w[illegible]rse than those with lo[illegible]er expr[illegible] of miR-203, suggesting th[illegible]e increased e[illegible]ion of miR-203 is a p[illegible] prognostic factor. [illegible]e Dual-Luciferase [illegible] repor[illegible]assay revealed that transfection [illegible]iR-2[illegible]mimic [illegible]gnificantly reduced the relati[illegible]se act[illegible] in pGL3-SOCS3-WT [illegible]sfect[illegible]K29[illegible]ells, but miR-203 mimic [illegible] affe[illegible]tive Luciferase activity in HE[illegible]3T cells [illegible]nsfected with pGL3-SOCS3-MU[illegible]dicating that there is a targeted regulati[illegible]p between miR-203 and SOCS3 [illegible]NA. In this study, the results of cell culture *in* [illegible] showed that compared with normal ovarian e[illegible]al IOSE80 cells, the expression of miR-203 [illegible] ovarian cancer HO8910 and SKOV3 cells was significantly increased, while the expression of SOCS3 mRNA was significantly decreased, which further suggested an increase in the expression level of miR-203 plays a role in [illegible] the expression of SOCS3 and promoti[illegible] of ovarian cancer. In this study, mi[illegible]3 inhibitor or miR-203 mimic was transfe[illegible]nto ovarian cancer cells cultured *in vitro*, and [illegible]iological effects of ovarian cancer c[illegible] were [illegible]d to further investigate the ro[illegible] miR-203 [illegible] an cancer. The results [illegible]is stu[illegible] showed transfection of miR[illegible] inhib[illegible] significantly up-regulated the exp[illegible]n [illegible]OCS3 [illegible] and protein in ovari[illegible]canc[illegible]s, and [illegible]reased the expression [illegible]p-JAK2 a[illegible]STA[illegible] protein. The cell pr[illegible]tion ability [illegible]gnificantly weakened [illegible]d [illegible]poptosis wa[illegible] significantly increased. On the [illegible] hand, transfection of miR-203 [illegible]imic overex[illegible]ng miR-203 promoted [illegible]liferation of ov[illegible]an cancer cells and [illegible]ced the apoptosis of cells, confirming that [illegible]-203 plays a[illegible]e as a cancer-promoting gene [illegible]arian cance[illegible] the study of the relationship b[illegible]n miR-20[illegible]nd ovarian cancer, the results of [illegible]y Shao et al[24] showed that abnormal [illegible]ssion of miR-203 is associated with poor prognosis of various tumors such as pancre[illegible]r, colorectal cancer, and ovarian cancer. [illegible] ratio for poor prognosis with ovarian cancer was 1.85 (95% CI 1.45 to 2.37). Azizmohammadi et al[12] found that the expression of miR-203 was significantly increased in tumor tissues of patients with ovarian cancer compared with adjacent tissues, and the expression of miR-203 was abnormally increased and was associated with FIGO stage (p=0.006), pathological grade (p=0.03). The high expression level of miR-203 was associated with poor prognosis (p<0.001). Wang et al[14] showed that the expression of miR-203 was significantly increased in tumor tissues of patients with ovarian cancer compared with adjacent tissues, and the expression of miR-203 was abnormally increased and associated with FIGO stage (p<0.001), pathological grade (p=0.02), lymph node metastasis (p<0.001), and recurrence (p<0.001), and the expression of miR-203 increased with the overall survival rate of patients (p<0.001) and no disease progression survival rate was associated with a decrease (p<0.001). Xiaohong et al[11] disclosed that the expression of miR-203 was significantly increased in tumor tissues of patients with ovarian cancer compared with adjacent tissues. Overexpression of miR-203 promoted proliferation and cloning formation, and migration of ovarian cancer cells Ovca429

and Ovca433. Down-regulation of miR-203 expression can attenuate the proliferation, clonality, and migration of ovarian cancer Ovca429, Ovca433 cells. The biological effect of miR-203 on ovarian cancer cells is by targeting the inhibition of Pyruvate Dehydrogenase E1 Beta Subunit (PDHB) gene expression and is achieved by regulating the glycolytic pathway of ovarian cancer cells. In this study, the expression of miR-203 was associated with ovarian cancer, and miR-203 plays a role in the role of oncogenes in ovarian cancer. This study combines the targeted regulatory relationship between miR-203 and SOCS3, revealing that miR-203 plays a role in regulating SOCS3 expression, affecting JAK-STAT pathway activity, and cell proliferation and apoptosis of ovarian cancer cells. This has not been reported in any domestic and foreign research. However, whether miR-203 regulates the biological effects of SOCS3 on ovarian cancer cells *in vivo* is unclear and needs to be confirmed by further animal experiments.

Conclusions

The increased expression of miR-203 a[illegible]he decreased expression of SOCS3 are relate[illegible] the pathogenesis of ovarian cancer. MiR-203 [illegible] inhibit the proliferation of ovaria[illegible]er cells b[illegible] targeting the inhibition of SO[illegible]sion and affecting the activity of JA[illegible]AT pa[illegible]ay.

Conflict of Intere[illegible]

The Authors declar[illegible]y have no con[illegible]nterests.

Refere[illegible]

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