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Original Article

Study on the role of miR-124 in exerting tumor suppressing effects on cervical cancer by regulating the Jak-Stat3 signaling pathway

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Abstract: The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway mediated activation of STAT3 plays critical roles in occurrence of multiple tumors. This study aimed to investigate whether microRNA-124 (miR-124) regulated STAT3 expression and proliferation/apoptosis of cervical carcinoma cells. Online prediction showed binding sites between miR-124 and 3'-UTR of STAT3 mRNA. Dual luciferase reporter gene assay confirmed targeted regulation between miR-124 and STAT3. Expression of miR-124, STAT3, p-STAT3 was compared among immortalized cervical epithelial cell line H8, Ect1/E6E7 and human cervical cancer cell lines SiHa and Caski. Flow cytometry tested cell proliferation and apoptosis. Cultured SiHa and Caski cells were transfected with miR-124 or si-STAT3, followed by flow cytometry for apoptosis and EdU staining for proliferation. miR-124 mimic significantly decreased the relative luciferase activity of cells transfected with STAT3 ($P<0.05$). Compared to H8 or Ect1/E6E7 cells, cervical cancer cells had significantly lower miR-124 expression, elevated STAT3 or p-STAT3 expression, accompanied by accelerated cell cycle and enhanced proliferation ($P<0.05$). Transfection of miR-124 mimic or si-STAT3 significantly suppressed STAT3 and p-STAT3 expression in cervical cancer cells ($P<0.05$), reduced cell proliferation, and promoted apoptosis. In conclusion, reduced miR-124 and increased STAT3 and p-STAT3 expression in cervical carcinoma cell lines HeLa and CaSki. Upregulation of miR-124 inhibited cell proliferation and increased apoptosis.

Keywords: miR-124, STAT3, cell apoptosis, cell proliferation, cervical carcinoma

Introduction

Cervical carcinoma (CC) is one commonly occurred female specific malignant tumor, and has been the second popular cancer in females only next to breast cancer [1], thus severely threatening public health of women [2, 3]. Human papillomavirus (HPV) infection is one critical pathogenic factor in CC pathogenesis. In recent years, increasing rate of HPV infection contributes to gradual increase of CC occurrence rate, plus younger age of patient population. Surgical resection is the major approach for early stage CC, whilst advanced stage patients mainly receive combined therapy including surgery, radiotherapy and chemotherapy. However, overall survival and prognosis of patients are still unfavorable. Therefore, the investigation of CC pathogenesis mechanism, and signaling pathway with abnormal changes, are of critical importance for improving chemotherapy efficiency, guideline of individualized treatment, and improvement of patient prognosis.

As one important transcriptional protein family of Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway, STAT3 plays a crucial role in tumor pathogenesis as an oncogene. When STAT3 expression or function is abnormally potentiated, consequently abnormal activation of JAK-STAT3 signaling pathway plays crucial modulatory effects in occurrence, progression, metastasis, and acquisition of drug resistance of various tumors including lung cancer, color cancer, pancreatic carcinoma and breast cancer [4-7]. Various studies showed the involvement of abnormally up-regulation or elevated expression of

STAT3 in CC occurrence [8, 9]. MicroRNAs (miRs) are endogenous non-coding small molecule single-stranded RNAs with about 22~25 nucleotides length, and can regulate target gene expression via complementary binding onto 3'-UTR of target gene mRNA to degrade or to inhibit gene translation. Although only occupying about 1% of total human genes, miR family can regulate up to one third of human gene expression [10]. Various studies showed that miR-124 was one important oncogenic gene, as its down-regulation has been found in various tumor tissues or cancer cells such as breast cancer, lung cancer and esophageal carcinoma [11-13]. Multiple studies have shown that CC cells had abnormally depressed miR-124 expression, indicating tumor suppressor role of miR-124 during CC pathogenesis [14]. This study thus aimed to investigate if miR-221 played a role in mediating SOCS3 expression, affecting JAK-STAT signal pathway activity and cancer cell biological behaviors.

Materials and methods

Major reagents and materials

Human immortalized cervical epithelial cell line H8, Ect1/E6E7, and human CC cell line SiHa, Caski were purchased from Xinzhou Biotech (China). RPMI 1640 culture medium and penicillin- streptomycin were purchased from Gibco (US). Fetal bovine serum (FBS) was purchased from Irvine Scientific (US). Total RNA extraction reagent TRNzol Universal was purchased from Tiangen Biochem (China). Lipofectamine 2000 was purchased from Invitrogen (US). PCR reagent PrimerScript RT reagent kit was purchased from Takara (China). MiR-124 mimic, miR-NC and EdU cell proliferation kit was purchased from RioBio (China). Si-NC and si-STAT3 were purchased from GE Pharmacon (US). Mouse anti-STAT3 and p-STAT3 antibody was purchased from Abcam (US). Rabbit anti-β-actin antibody was purchased from Abnova (US). HRP conjugated secondary antibody was purchased from Sango Bio (China). Dual luciferase reporter plasmid pGL3 and dual luciferase reporter assay system were purchased from Promega (US).

Cell culture

H8, Ect1/E6E7, SiHa and Caski cells were all cultured in RPMI 1640 medium containing 10% FBS, and were cultured in a 37°C incubator with 5% CO_2. Those cells at log-growth phase with satisfactory status were used for further assays.

Construction of luciferase reporter gene assay

Using HEK293T cell RNA as the template, PCR amplification was performed to include 3'-UTR of STAT3 mRNA containing miR-124 binding sites. At 5'-terminus of forward and reverse primers, restriction sites of Sac I and Hind III enzymes plus protective bases were added. Primer sequences were: Forward, 5'-CGAGC TCGCG ACAGT CTGAG ACTCT GTCT-3'; Reverse, 5'-CCAAG CTTGA TAAGG CACCC ACAGA AACAA-3' (Sac I and Hind III enzymatic digestion sites were highlighted by underlines, with protective bases were added in front). Amplification products were within 851 bp to 2017 bp range of 3'-UTR of STAT3 gene. PCR amplification system consisted of 10 μL 2XKOD buffer, 4 μL dNTP (2 mM), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.4 μL KOD FX, 2 μL template DNA and 3.6 μL ddH_2O. PCR amplification parameters were: 95°C for 10 minutes, followed by 30 cycles each consisting of 94°C 30 seconds, 58°C 30 seconds and 72°C 30 seconds, and ended with 72°C for 10 minutes. After PCR amplification, Sac I and Hind III were used for dual-enzymatic digestion on PCR products and pGL3 empty plasmids at 37°C for 4 hours. Purification products were extracted by 1.5% agarose gel electrophoresis, and were ligated with plasmid for 16°C overnight incubation. Ligated products were used to transform DH5α competent cells, which were inoculated onto penicillin-containing plate for 37°C overnight incubation. A single positive clone was selected and incubated at 37°C overnight. Plasmids were extracted for sequencing of target genes, and the plasmid with successful gene insertion was named as pGL-SOCS3-WT. Using wild type plasmid as the template, binding sites between miR-124 and 3'UTR of STAT3 were mutated into nonsense sequence. Site-directed mutagenesis primers were designed: Forward, 5'-CGAGC TCGCG ACAGT CTGAG ACTCT GTCT-3'; Reverse primer, 5'-CCAAG CTTGA TTACG GAGCC ACAGA AACAA-3' (mutated sequence was highlighted by underlines). The mutated plasmid was named as pGL-STAT3-MUT prepared using the same procedure as those in wild type plasmids. Lipofectamine 2000 was used to co-transfect pG-

L3-STAT3-WT or pGL3-STAT3-MUT with miR-124 mimic or miR-NC into HEK293T cells. After 48 hour incubation, the dual luciferase reporter assay system kit was used to test relative luciferase activity.

Cell transfection and grouping

Cultured SiHa and Caski cells were divided into four transfection groups: miR-NC transfection group, miR-124 mimic transfection group, si-NC transfection group, and si-STAT3 transfection group. These nucleotide sequences and Lipofectamine 2000 were diluted in Opti-MEM medium for 5 minutes room temperature incubation. These nucleotide sequences were mixed gently with Lipofectamine 2000 for 30 minute room temperature incubation. SiHa and Caski cells were removed for original medium, and washed twice in PBS to remove serum. Opti-MEM serum-fee culture medium was used to add transfection mixture. Cells were then incubated for 6 hours, and RPMI 1640 culture medium containing 10% FBS and 1% penicillin-streptomycin was used. After 72 hour continuous incubation, cells were digested by trypsin and were collected for further assays. Nucleotide sequences used for transfection were: si-STAT3 sense, 5'-CAUCU GCCUA GAUCG GCUA-3'; si-STAT3 anti-sense, 5'-UAGCC GAUCU AGGCAG AUG-3'; si-NC sense, 5'-UUCUC CGAAC GUGUC ACGU-3'; si-NC anti-sense, 5'-ACGUG ACACG UUCGG AGAA-3'.

qRT-PCR

PrimeScript RT reagent Kit was used to perform qRT-PCR assay for measuring relative gene expression level, using RNA samples extracted by total RNA kit. Reverse transcription system was as follows: oligdT Primer (50 μM), 0.5 μL; Random 6 mers (100 μM), 0.5 μL; PrimeScript RT Enzyme Mix, 0.5 μL; RNA, 1.0 μg; 5× PrimeScript Buffer, 2 μL; RNase Free dH_2O, Up to 10.0 μL. qPCR amplification system was as follows: SYBR Fast qPCR Mix, 10.0 μL; Forward Primer (10 μM), 0.8 μL; Reverse Primer (10 μM), 0.8 μL; cDNA, 2.0 μL; RNase Free dH_2O, 6.4 μL.

Reaction conditions were: 95°C pre-denature for 10 minutes, followed by 40 cycles each consisting of 95°C denature for 10 seconds, 60°C annealing for 20 seconds, and 72°C elongation for 15 seconds. PCR was performed on Bio-Rad CFX96 Real Time PCR Detection System. Primer sequences were: miR-124P_F: 5'-CGGTA AGGCA CGCGG TGA-3'; miR-124P_R: 5'-AGTGC GAACT GTGGC GAT-3'; U6P_F: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; U6P_R: 5'-GGAAC GCTTC ACGAA TTTG-3'; STAT3P_F: 5'-ATCAC GCCTT CTACA GACTG C-3'; STAT3P_R: 5'-CATCC TGGAG ATTCT CTACC ACT-3'; β-actinP_F: 5'-GAACC CTAAG GCCAA C-3'; β-actinP_R: 5'-TGTCA CGCAC GATTT CC-3'.

Western blot

Cells were lysed by SDS lysis buffer, and were boiled in 100°C for 5 minutes. Protein concentrations were measure, and 40 μg samples were loaded and separated using 8%~10% SDS-PAGE separating gel and 5% condensing gel. Proteins were transferred to PVDF membrane, which was blocked in 5% defatted milk powder. Primary antibody (STAT3 at 1:1000, p-STAT3 at 1:3000, β-actin at 1:6000) was added for 4°C overnight incubation, followed by three times of PBST washing. HRP conjugated secondary antibody (1:20000 dilution) was added for room temperature, followed by three times of PBST washing. ECL developing reagent was added for 1~3 minute reaction, and the membrane was exposed, developed, and the film was scanned for data analysis.

Assay for cell apoptosis

Cells were digested and collected by trypsin, and were re-suspended in Binding Buffer. Then, 5 μL Annexin V-FITC and 5 μL PI solution were added. Cell apoptosis was then measured by Beckman Coulter FC500 MCL/MPL flow cytometry system.

Flow cytometry for cell proliferation

Cells from all transfection groups were re-suspended, and were incubated in 10 μM EdU for 2 hours, followed by 48 hour continuous incubation. Cells were digested in trypsin, centrifuged, and fixed. Reaction buffer with Alexa Fluor 488 labels was added for 30 minute incubation in the dark at room temperature. After centrifugation and washing, cell proliferation was measured by Beckman Coulter FC 500 MCL/MPL flow cytometry.

Flow cytometry for cell cycle

Cells were digested in trypsin and were collected. After washing in PBS, 70% ethanol was ad-

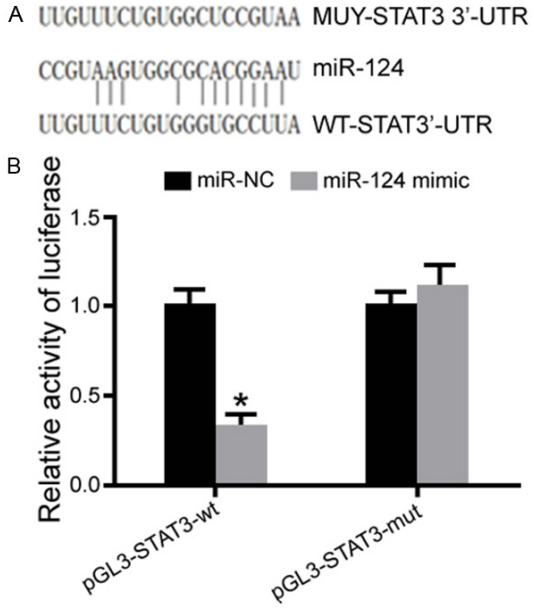


Figure 1. Targeted relationship between miR-124 and STAT3 mRNA. A. Function site between miR-124 and 3'-UTR of STAT3 mRNA; B. Dual luciferase gene reporter assay. **, P<0.05 comparing to miR-NC group.

ded for fixation at -20°C for 60 minutes. Cells were then centrifuged in PSB twice for washing. PI buffer containing RNase A was then added for 4°C dark incubation for 30 minutes. Cell cycle was measured on Beckman FC500 MCL/MPL flow cytometry system.

Statistical analysis

SPSS 18.0 was used for data analysis. Measurement data are presented as mean ± standard deviation (SD). Student t-test was used for comparison of measurement data between groups. A statistical significance was defined when P<0.05.

Results

Targeted functional relationship between miR-124 and STAT3 mRNA

Bioinformatics analysis showed the existing of complementary binding sites between miR-124 and 3'-UTR of STAT3 mRNA (**Figure 1A**). Dual luciferase gene reporter assay showed that, compared to miR-NC transfection group, miR-124 mimic transfection significantly reduced the relative luciferase activity of cells transfected with wild type STAT3 (P<0.05) without affecting the luciferase activity of cells transfected with mutant STAT3 (**Figure 1B**), suggesting that miR-124 could target 3'-UTR of STAT3 mRNA.

Down-regulation of miR-124 and up-regulation of STAT3 in CC cells

qRT-PCR results showed that, comparing to H8 or Ect1/E6E7 cells, miR-124 expression in SiHa and Caski cells was significantly suppressed, while STAT3 mRNA level was up-regulated (P<0.05) (**Figure 2A**). Western blot results showed remarkably higher STAT3 and p-STAT3 protein expression in SiHa and Caski cells compared to those in H8 and Ect1/E6E7 cells (P< 0.05) (**Figure 2B**). Cell cycle assay showed that SiHa and Caski cells had significantly higher ratio of S phase and G2/M phase cells than H8 or Ect1/E6E7 cells (P<0.05), whilst whilst G0/G1 cell ratio was significantly lower (P<0.05) (**Figure 2C**; **Table 1**). Flow cytometry results showed that SiHa and Caski cells had significantly higher EdU positive rate than H8 or Ect1/E6E7 cells (P<0.05), indicating potentiated proliferation potency (**Figure 2D**).

miR-124 up-regulation weakened proliferation potency of SiHa and Caski cells, and induced cell apoptosis

The transfection of miR-124 mimic and/or si-STAT3 significantly suppressed expression of STAT3 and p-STAT3 in SiHa cells (P<0.05) (**Figure 3A**) and Caski cells (**Figure 3B**), and weakened cell proliferation (**Figure 3C**), in addition to elevated cell apoptosis (**Figure 3D**).

Discussion

The JAK-STAT signal pathway is widely activated in various mammalian tissues, and is involved in the regulation of multiple biological processes including cell survival, proliferation, cell cycle, apoptosis, migration and invasion [15, 16]. STATs are important transcriptional factors downstream of JAK signaling, and mainly exert transcriptional activation in the nucleus and promote expression of multiple genes, thus facilitating cell proliferation and antagonizing cell apoptosis, thus is closely correlated with occurrence of multiple tumors. Cytokine and growth factor work as the ligand for binding with intracellular receptor, which forms homo- or hetero-dimers, and phosphorylates JAK for activation. Such activated JAK can phosphory-

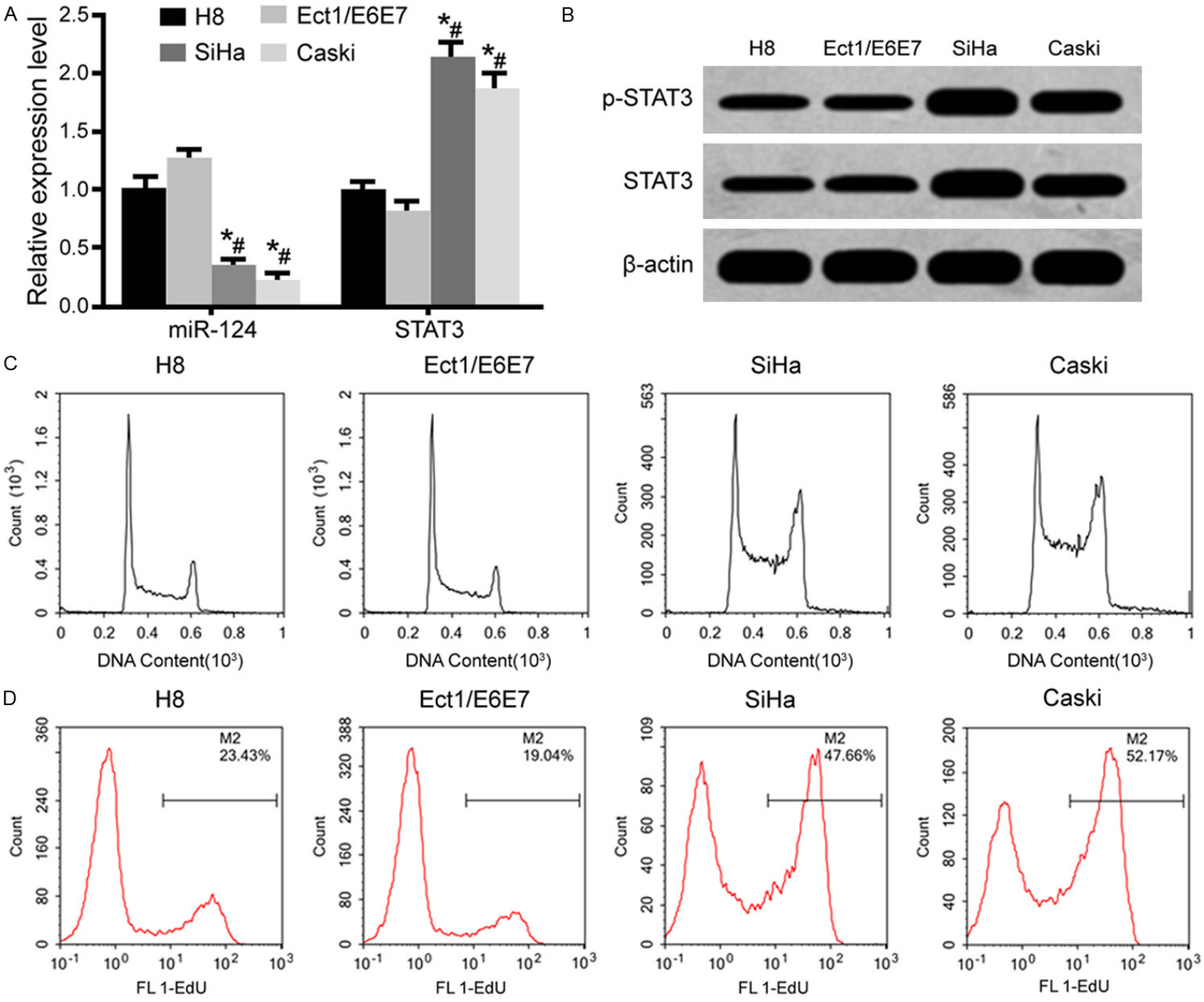

A
H8
Ect1/E6E7
SiHa
Caski
Relative expression level
2.5
2.0
1.5
1.0
0.5
0.0
*#
*#
*#
*#
miR-124
STAT3
B
H8
Ect1/E6E7
SiHa
Caski
p-STAT3
STAT3
β-actin
C
H8
Ect1/E6E7
SiHa
Caski
Count (10³)
Count
DNA Content(10³)
D
H8
Ect1/E6E7
SiHa
Caski
M2
23.43%
M2
19.04%
M2
47.66%
M2
52.17%
Count
FL 1-EdU

Figure 2. miR-124 down-regulation and STAT3 up-regulation in CC cells. A. qRT-PCR for gene expression; B. Western blot for protein expression; C. Flow cytometry for cell cycle; D. Flow cytometry for cell proliferation. *, P<0.05 compared to H8 cells; #, P<0.05 compared to Ect1/E6E7 cells.

Table 1. Cell cycle distribution of all cell lines

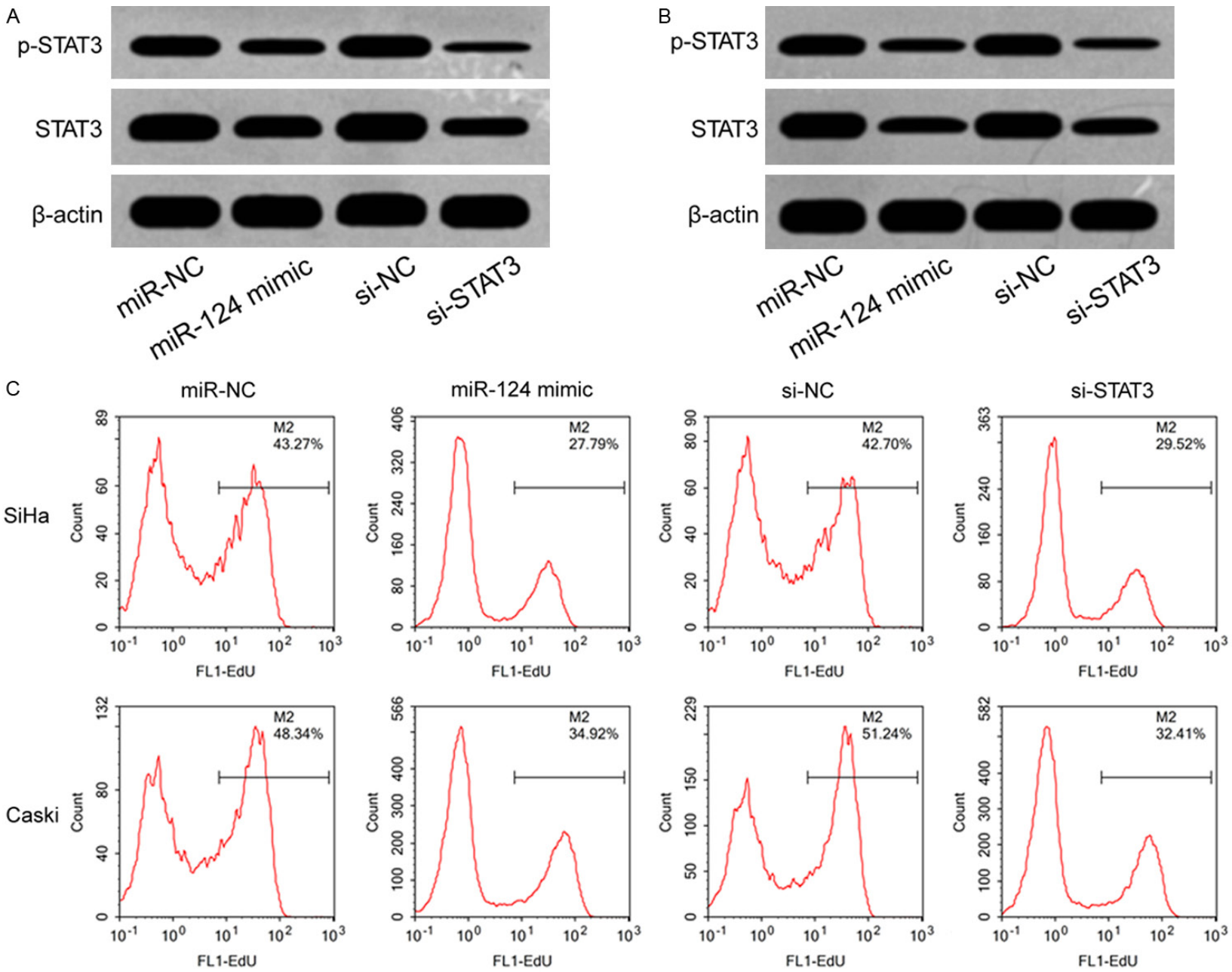
	G0/G1	S	G2/M
H8	66.5±5.1	18.3±1.6	15.2±1.4
Ect1/E6E7	69.2±4.9	16.7±1.4	14.1±1.6
SiHa	49.1±3.9*,#	27.6±2.9*,#	23.3±1.9*,#
Caski	52.3±4.6*,#	25.3±2.3*,#	22.4±2.8*,#

*, P<0.05 compared to H8 cells; #, P<0.05 compared to Ect1/E6E7 cells.

late tyrosine residue on receptor molecules, facilitating STAT binding with tyrosine phosphorylating sites of receptor complex via SH2 structural domain. At that time, JAK kinase has spatial proximity towards STAT and phosphorylates hydroxy-tyrosine residue. Such phosphorylated STAT is detached from receptor complex, forming dimers for translocation into the nucleus, where it acts on specific DNA sequence, thus modulating gene transcription and expression [15, 17, 18]. STAT protein family consists of STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6, among which STAT3 and STAT5 are most closely correlated with human tumor pathogenesis. STAT3 has prominent expression and critical function [2]. When STAT3 expression or function is abnormally enhanced, it can induce aberrant activation of JAK-STAT3 signaling pathway, and is thus closely correlated with occurrence, progression, metastasis, and drug resistance of multiple tumors [4-7]. Various studies found that abnormally elevated expression or enhanced activity of STAT3 was closely correlated with growth, infiltration, and metastasis and drug resistance of CC tissues [8, 9]. Bioinformatics analysis revealed the existence of targeted binding sites between miR-221 and 3'-UTR of SOCS3 mRNA. This study thus investigated if miR-221 played a role in mediating SOCS3 expression, affecting JAK-STAT3 signal pathway activity, and CC cell biological behavior.

Dual luciferase gene reporter assay showed that, compared to miR-NC group, miR-124 mimic transfected CC cells showed significantly depressed relative luciferase activity, while miR-124 mimic had no effect on relative luciferase activity in CC cells transfected with pGL3-STAT3-mut. These results suggest a targeted regulatory relationship between miR-124 and STAT3 mRNA. Comparing to H8 and Ect1/E6E7 cells, CC cell lines SiHa and Caski showed significantly suppressed miR-124 expression, and elevated expression of STAT3 and p-STAT3, accompanied with enhanced proliferation potency and accelerated cell cycle progression. Dong et al. found that, comparing to normal endometrial epithelial cells, CC cell lines SiHa and HeLa had elevated methylation level in miR-124 gene promoter, plus significantly lower miR-124 expression. Comparing to tumor adjacent tissues, CC tissues had abnormally decreased miR-124 expression, which was positively correlated with 5-year overall survival and progression-free survival of patients [19]. Jimenez-Wences et al. found that, comparing to early stage cervical squamous epithelial lesions, CC tumor tissues had significant hyper-methylation of miR-124 gene promoter region, plus remarkably lower miR-124 expression [20]. In this study, CC cells showed lower miR-124 expression compared to non-tumor cervical epithelial cells, indicating that miR-124 was candidate tumor suppressor gene of CC. This is consistent with Done et al. [19] and Jimenez-Wences et al. [20]. Ramirez et al. showed that compared to non-tumor immortalized epithelial cell line HaCaT, CC cell lines HeLa, SiHa and C-33A showed remarkably elevated STAT3 expression [21], supporting our observation.

Further study showed that transfection of miR-124 mimic and/or si-STAT3 significantly suppressed STAT3 and p-STAT3 expression in SiHa and Caski cells, weakened cell proliferation and enhanced apoptosis. Liu et al. showed that MALAT1 could exert regulatory function on proliferation, invasion, and apoptosis of CC cells via MALAT1-miR-124-RBG2 axis. Specifically, MALAT1 can up-regulate RGB2 expression, via suppressing miR-124 expression, thus facilitating CC cell proliferation and invasion, and decreasing apoptosis [22], thus demonstrating the tumor suppressor role of miR-124 in CC. Wan et al. found that miR-124 could weaken proliferation, invasion and epithelial mesenchymal transition (EMT) of CC cell lines HeLa and C33A via targeted inhibition on AmotL1 expression [23]. Dong et al. found that methylation


A
p-STAT3
STAT3
β-actin
miR-NC
miR-124 mimic
si-NC
si-STAT3
B
p-STAT3
STAT3
β-actin
miR-NC
miR-124 mimic
si-NC
si-STAT3
C
miR-NC
miR-124 mimic
si-NC
si-STAT3
SiHa
Caski
Count
FL1-EdU
M2
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29.52%
48.34%
34.92%
51.24%
32.41%

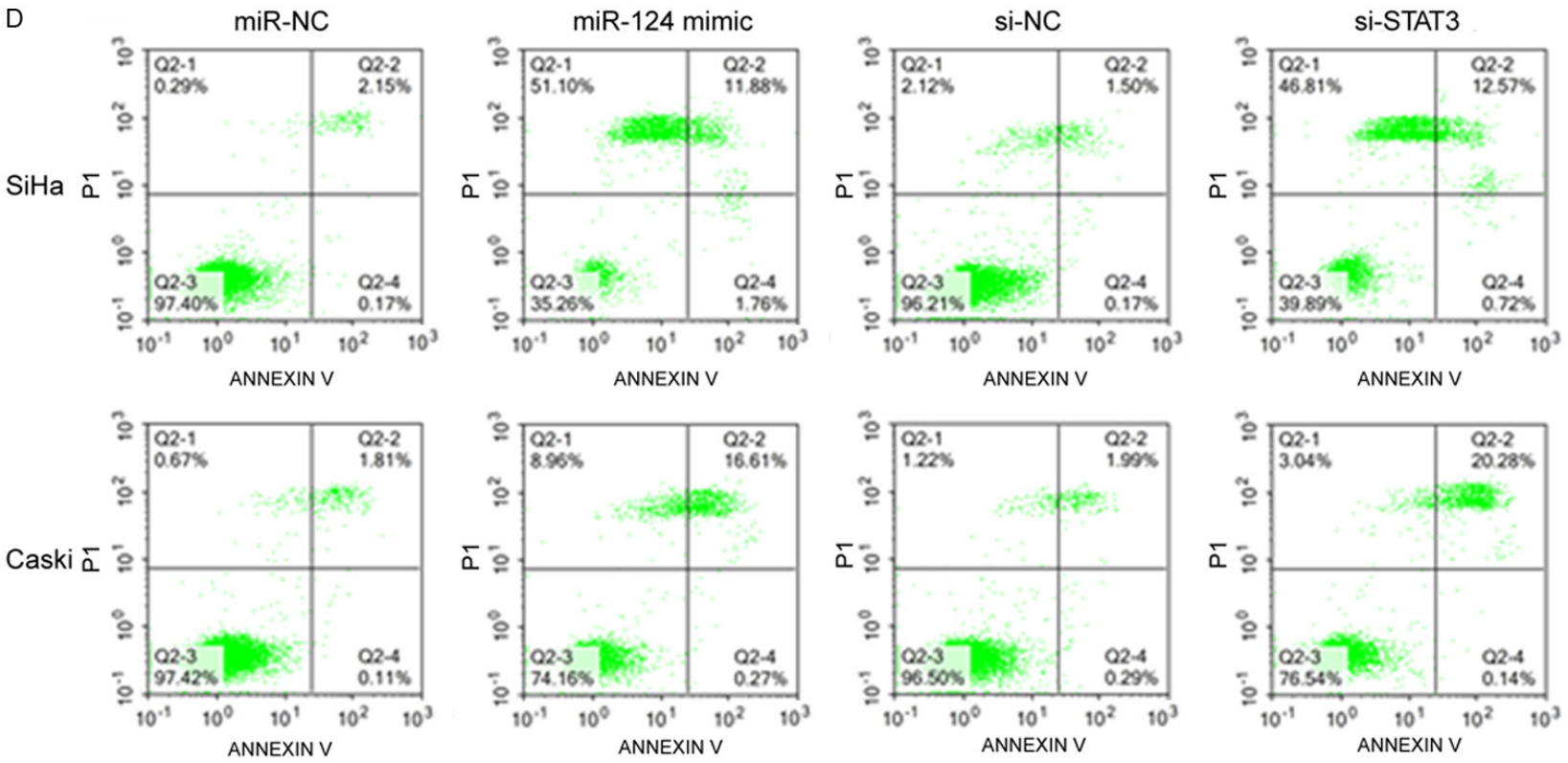

Figure 3. miR-124 up-regulation weakened proliferation potency of SiHa and Caski cells, and induced cell apoptosis. A. Western blot for protein expression in SiHa cells; B. Western blot for protein expression in Caski cells; C. Flow cytometry for cell proliferation; D. Flow cytometry for cell apoptosis.

inhibitor Zebularine treatment significantly enhanced miR-124 expression in CC cells SiHa and HaLa, whose expression level of target gene iASPP was remarkably decreased, accompanied with weakened proliferation and invasion potency of these cells [19]. Wilting et al. showed that after using demethylation agent to suppress miR-124 methylation, miR-124 expression level was significantly elevated, whilst its target gene IGFBP7 was down-regulated, accompanied by prominent inhibition on cell proliferation or migration of SiHa and CaSki [24], indicating that miR-124 exerted tumor suppressor role on CC via targeted regulation on IGFBP7. Fan et al. showed that STAT3 up-regulation was correlated with enhanced proliferation, decreased apoptosis and chemotherapy drug resistance of CC cell lines HeLa and CaKsi, while STAT3 down-regulation could weaken proliferation of CC cells, induce cell apoptosis, and decrease drug resistance [25]. Wang et al. found that STAT3 up-regulation exerted certain roles in enhancing stem cell property of CC cells, and using siRNA to suppress STAT3 expression could weaken tumor stem cell behaviors and malignant biological property of Siha cells [25]. This study analyzed expressional profiles of miR-124 and STAT3 in CC cells, and revealed important roles of miR-124 in mediating STAT3 expression and in affecting proliferation and apoptosis of CC cells HeLa and CaSki, which have not been previously reported by other groups. Further studies can be performed to analyze expressional profile of miR-124 and STAT3 in CC tissues, in order to investigate whether miR-124 mediated regulation on STAT3 is active in CC patient tumor tissues, which was not reported in this study.

Conclusion

miR-124 up-regulation significantly suppresses STAT3 and p-STAT3 expression in CC cell lines HeLa and CaSki, weakens cell proliferation potency, and induces cell apoptosis.

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Disclosure of conflict of interest

None.

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