

miR-29c is downregulated in the ectopic endometrium and exerts its effects on endometrial cell proliferation, apoptosis and invasion by targeting c-Jun

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Abstract. Endometriosis is a prevalent and complex gynecological disease which affects 10% of women of reproductive age. Certain studies have suggested that a substantial number of microRNAs (miRNAs or miRs) are aberrantly or differentially expressed in the ectopic endometrium. To date, to the best of our knowledge, there is no report available on the role of miR-29 in the endometrium. In this study, we investigated the expression of the miR-29 family in the endometrium samples from women without endometriosis, as well as in paired ectopic and eutopic endometrium samples by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The results revealed that miR-29c was differentially expressed in the paired eutopic and ectopic endometrium samples. In addition, c-Jun was differentially expressed in the ectopic and eutopic endometrial tissues as determined by western blot analysis. Furthermore, the role of miR-29c in endometrial cell proliferation, invasion and apoptosis was examined *in vitro*. The results revealed that miR-29c exerted its effects on endometrial cells by suppressing cell proliferation and invasion, as well as promoting cell apoptosis. Furthermore, it was found that c-Jun was a novel target of miR-29c, and c-Jun reversed the effects of miR-29c on the proliferation, invasion and apoptosis of endometrial cells. To the best of our knowledge, this study is the first to identify miR-29c as a suppressor of endometriosis. Taken together, our results suggest that miR-29c exerts its effects on endometrial cell proliferation, apoptosis and invasion by inhibiting the expression of c-Jun. Our data

may provide a novel potential therapeutic target for the treatment of endometriosis.

Introduction

Endometriosis is a prevalent and complex gynecological disease which affects 10% of women of reproductive age (1), and is characterized by the growth of endometrial glandular epithelial and stromal cells outside the uterine cavity. Women of reproductive age with endometriosis often suffer from painful menstrual periods, chronic pelvic pain and infertility. This disease is multifactorial and factors, such as hormones, immune response and the environment may contribute to the susceptibility and progression of endometriosis (2-4). In the majority of cases, the spread of extrauterine endometrial tissue is due to retrograde menstruation and lymphatic dissemination (5).

microRNAs (miRNAs or miRs) are small non-coding RNAs, which are now known to play essential roles in the regulation of gene expression for cell proliferation and apoptosis, cell cycle, immune response and inflammation (6,7). The deregulation and aberrant expression of these molecules has been implicated in a variety of human diseases, including cancer (8,9).

Endometriosis displays a benign morphology; however, endometriotic cells have an invasive potential which is characterized by malignant cells (10). To date, to the best of our knowledge, there is no report available on the role of miR-29 in the endometrium. In the present study, we investigated the expression of miR-29a, miR-29b, miR-29c in endometrium samples from women without endometriosis, as well as in paired ectopic and eutopic endometrium samples. Furthermore, the role of miR-29c in endometrial cell proliferation, invasion and apoptosis was examined *in vitro*, and a novel target through which miR-29c exerts its effects on endometrial cells was identified.

Materials and methods

Tissue samples. The procedures for the collection of the tissue samples were approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University, Urumqi,

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China. All patients signed an informed consent form in compliance with the code of ethics of the World Medical Association (Declaration of Helsinki). Paired eutopic and ectopic endometrium samples were collected from 20 women with endometriosis who underwent laparoscopic surgical procedures at the First Affiliated Hospital of Xinjiang Medical University. The patients were aged 25 to 42 years, had regular menstrual cycles and had not received hormonal therapy for at least 3 months prior to surgery. A total of 10 samples of endometrium from women with hysteromyoma were used as the controls. The tissues were from the proliferative phase of the menstrual cycle. After collection, the tissue samples were immediately frozen and kept in liquid nitrogen for further analysis.

Cell culture and transfection. The CRL-7566 endometriosis cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). This cell line was established from a benign ovarian cyst obtained from a patient with endometriosis. The cells were isolated from both the inner and outer surfaces of the cyst. The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA). The cells were grown at 37°C in 5% CO₂. The miR-29c mimic, miR-29c inhibitor and negative control (NC), mutant and wild-type c-Jun 3'UTR, as well as the c-Jun overexpression plasmid and the shRNA plasmid were transfected into the cells using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions and incubated at 37°C for 24 h.

Luciferase assay. The targets of miR-29c were predicted by miRanda (<http://www.microrna.org/>) and c-Jun was identified to be a target of miR-29c. The sequences of human wild-type c-Jun 3' untranslated region (3'UTR) and mutant c-Jun 3'UTR were then cloned into the pGL3-control luciferase reporter vector (Promega, Madison, WI, USA). c-Jun 3'UTR reporter plasmids were co-transfected with the miR-29c mimic or negative control (NC) into the cells. pRL Renilla luciferase reporter vector was used as the internal control in each assay. Following transfection, cell lysates were collected and luciferase activity was measured using the Dual-Luciferase Reporter assay system (Promega).

MTT assay. MTT assay was used to evaluate cell proliferation. The cells were seeded into 96-well culture plates at a density of 1x10⁴ cells/well. Following transfection, 10 µl MTT solution (0.5 mg/ml; Beyotime, Shanghai, China) were added to each well followed by incubation for 4 h. After dissolving the MTT formazan with DMSO (Sigma, St. Louis, MO, USA), cell proliferation was determined by reading the plates at 570 nm on a Multiskan Ascent 354 microplate reader (Thermo Labsystems, Waltham, MA, USA).

Cell invasion assay. Cell invasion assay was performed using Matrigel-coated Transwell plates (Corning, New York, NY, USA). Cells in serum-free medium at a final density of 5x10⁴ cells/ml were added to the upper chambers and incubated at 37°C for 12 h, and cell medium containing 10% FBS was added to the lower chambers. Following incubation, non-invaded cells were removed using cotton swabs, and the invaded cells were fixed in 95% ethanol and stained with hematoxylin. The number

of invaded cells was determined by counting the stained cells under an inverted microscope (TS100; Nikon, Tokyo, Japan).

Flow cytometry (FCM). Cell apoptosis was assessed by FCM. After washing with phosphate-buffered saline (PBS), the cells were resuspended in binding buffer and stained with Annexin V and propidium iodide (PI) (Kaiji Biological Inc., Nanjing, China) following the manufacturer's instructions. Positive cells were detected and quantified by FCM (BD FACSaria; BD Biosciences, Franklin Lakes, NJ, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The miRNAs were isolated using a miRNeasy Mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania) and quantitative (real-time) PCR was carried out on a 7900 Real-Time PCR System using a SYBR-Green PCR kit (both from Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The results were analyzed using the comparative method following normalization of the expression values to U6.

Western blot analysis. Protein samples were extracted using a total protein extraction kit (Kaiji Biological, Inc.) and the protein concentration was determined using the BCA kit (Beyotime). The samples were separated in 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat milk overnight at 4°C. After washing with PBS, the membranes were incubated with primary antibody [mouse monoclonal to c-Jun (ab119944), mouse monoclonal to β-actin (ab8226); Abcam, Cambridge, MA, USA] overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (BA1050; Boster, Wuhan, Hubei, China) for 1 h at room temperature. The ECL detection kit (Pierce, Rockford, IL, USA) was used to detect the proteins.

Statistical analysis. Data were presented as the means ± standard deviation (SD). The two-tailed Student's t-test was used to statistically analyze data between 2 groups. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Differential expression of miR-29 family in tissue samples. The differential expression of miR-29a, miR-29b, miR-29c in the endometrium samples from women without endometriosis, as well as in the eutopic and ectopic endometrium samples was determined by RT-qPCR. The results revealed that the expression of miR-29c progressively decreased from the samples of endometriosis to the those of the eutopic and ectopic endometrium, and the expression of miR-29c between the eutopic and ectopic endometrium was differed significantly (P<0.01). However, the expression levels of miR-29a and miR-29b did not differ significantly between the 3 groups (Fig. 1).

Differential expression of c-Jun in tissue samples. The expression of c-Jun in the endometrium samples from women without

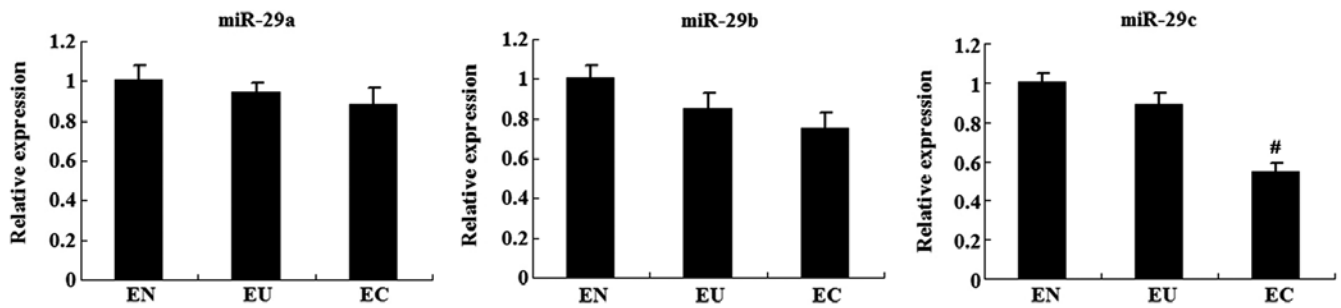


Figure 1. Differential expression of the miR-29 family in endometrial tissues determined by RT-qPCR. #P<0.01 compared with the EU group. EN, samples from women without endometriosis; EU, eutopic endometrium; EC, ectopic endometrium.

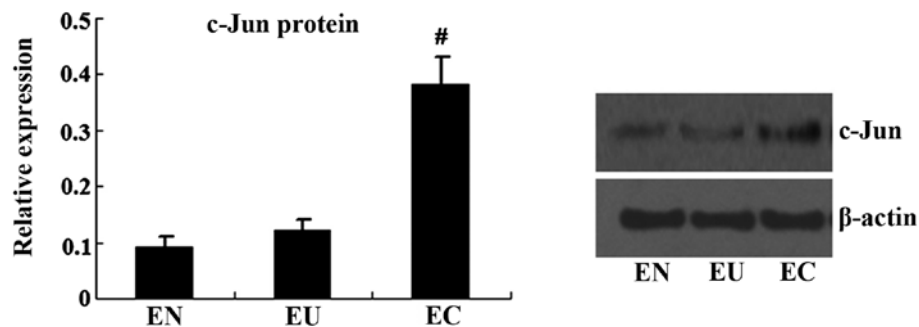


Figure 2. Differential expression of c-Jun in tissues determined by western blot analysis. #P<0.01 compared with the EU group. EN, samples from women without endometriosis; EU, eutopic endometrium; EC, ectopic endometrium.

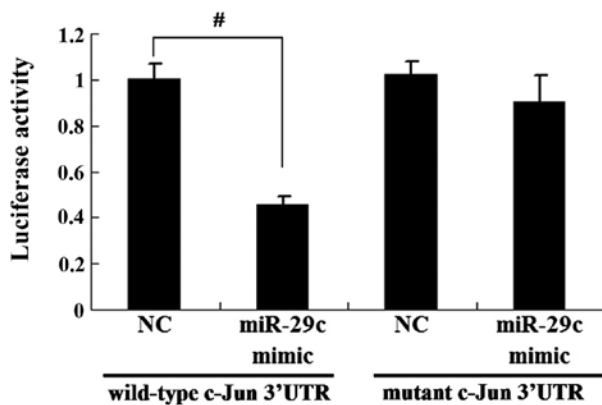


Figure 3. c-Jun is a direct target of miR-29c as determined by luciferase reporter assay. #P<0.01. NC, negative control.

endometriosis, as well as in the eutopic and ectopic endometrium samples was examined by western blot analysis. There was no statistically significant difference in c-Jun expression between the group without endometriosis and the eutopic group (Fig. 2). However, the protein expression level of c-Jun was significantly higher in the ectopic group compared with the eutopic group (P<0.01).

c-Jun is directly regulated by miR-29c. In order to determine whether c-Jun is a direct target of miR-29c, wild-type c-Jun-3'UTR or mutant c-Jun-3'UTR were co-transfected with the miR-29c mimic or NC. Luciferase assay revealed that transfection with the miR-29c mimic significantly inhibited the luciferase activity of wild-type c-Jun-3'UTR (P<0.01); however, the lucif-

erase activity of mutant c-Jun-3'UTR did not differ statistically following transfection with the miR-29c mimic or NC (Fig. 3).

Furthermore, the effect of miR-29c on c-Jun expression was examined by western blot analysis. The results from RT-qPCR revealed that miR-29c expression was significantly upregulated in the miR-29c mimic-transfected group and downregulated in the miR-29c inhibitor-transfected group (Fig. 4A). Western blot results revealed a significantly decreased expression of c-Jun in the cells transfected with the miR-29c mimic; however, the expression of c-Jun was increased in the cells transfected with the miR-29c inhibitor (Fig. 4B).

Expression of miR-29c and c-Jun following transfection with the c-Jun overexpression or shRNA plasmid. The open reading frame (ORF) clone of homo c-Jun was subcloned into the pcDNA3.1 vector to induce the overexpression of c-Jun. We suppressed c-Jun expression using a specific shRNA plasmid. Transfection with the c-Jun overexpression plasmid did not alter the increase in miR-29c expression in the miR-29c mimic-transfected cells (Fig. 5A). In addition, the c-Jun shRNA plasmid did not alter the decrease in miR-29c expression in miR-29c inhibitor-transfected cells (Fig. 5B).

Cells transfected with the miR-29c mimic and c-Jun overexpression plasmid showed a significantly increased protein expression of c-Jun compared with the cells transfected with the miR-29c mimic only (Fig. 6A). However, transfection with c-Jun shRNA significantly downregulated the expression of c-Jun in the cells transfected with the miR-29c inhibitor (Fig. 6B).

c-Jun reverses the miR-29c-induced suppression of cell proliferation and invasion. In order to examine the effects of

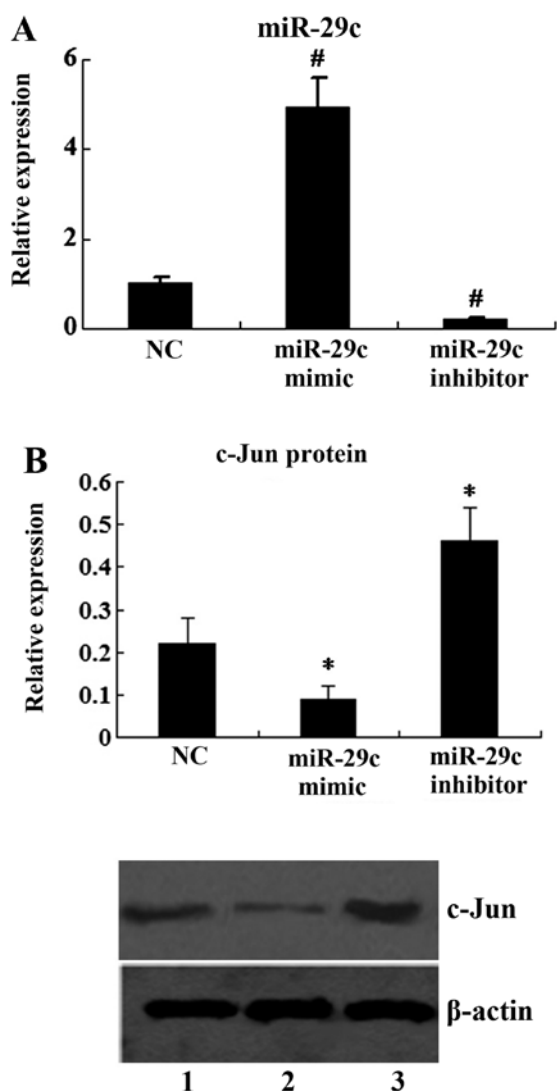


Figure 4. c-Jun expression is regulated by miR-29c. (A) mRNA expression of miR-29c following transfection with the miR-29c mimic or the miR-29c inhibitor in CRL-7566 cells. (B) Protein expression of c-Jun following transfection with the miR-29c mimic or the miR-29c inhibitor in CRL-7566 cells. Lane 1, NC; lane 2, miR-29c mimic; lane 3, miR-29c inhibitor. * $P < 0.05$ and # $P < 0.01$ compared with the NC group. NC, negative control.

miR-29c on cell proliferation, the miR-29c mimic or inhibitor were transfected into the cells, and MTT assay was used to analyze cell proliferation. Compared with the NC-transfected cells, the miR-29c mimic-transfected cells proliferated at a significantly lower rate (Fig. 7A). By contrast, transfection with the miR-29c inhibitor promoted cell growth (Fig. 7B).

Furthermore, the overexpression of c-Jun reversed the inhibitory effects of the miR-29c mimic on cell proliferation (Fig. 7A). Consistent with these results, transfection with c-Jun shRNA reversed the inductive effects of the miR-29c inhibitor on cell proliferation (Fig. 7B).

Cell invasion ability was determined by Transwell-Matrigel assay. Transfection with the miR-29c mimic resulted in significantly decreased cell invasion in comparison to transfection with NC; however this effect was reversed by transfection with the c-Jun overexpression plasmid (Fig. 8A). As expected, transfection with the miR-29c inhibitor significantly increased

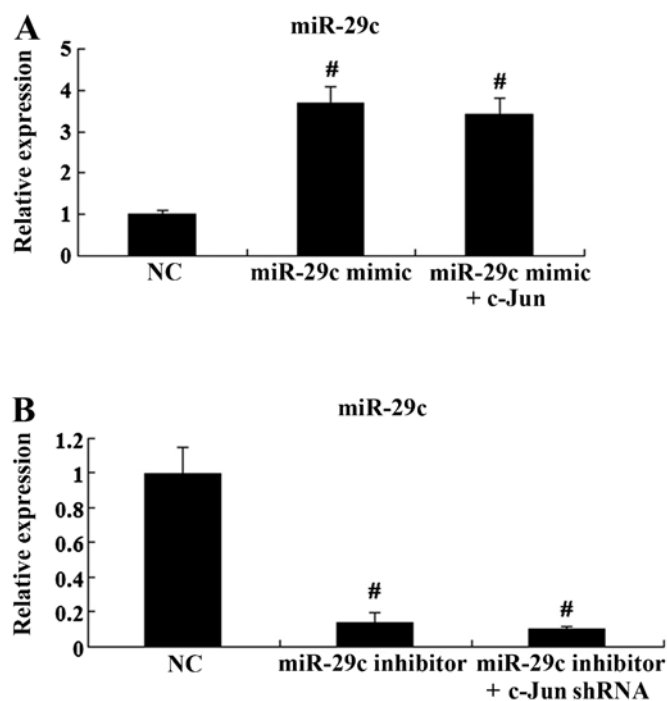


Figure 5. Expression of miR-29c mRNA following transfection with the miR-29c mimic/inhibitor and c-Jun overexpression/shRNA plasmid in CRL-7566 cells. (A) c-Jun overexpression plasmid did not alter the increase in miR-29c expression in miR-29c mimic-transfected cells. (B) c-Jun shRNA plasmid did not alter the decrease in miR-29c expression in miR-29c inhibitor-transfected cells. # $P < 0.01$ compared with the NC group. NC, negative control.

cell invasion ability, which was reversed by transfection with c-Jun shRNA (Fig. 8B).

c-Jun reverses the miR-29c-mediated induction of cell apoptosis. FCM was used to examine the cell apoptotic rates. The results revealed that compared with the cells transfected with NC, the miR-29c mimic-transfected cells showed an increased cell apoptotic rate, while transfection with miR-29c inhibitor had the opposite effect. The overexpression of c-Jun reversed the increase in the cell apoptotic rate induced by the transfection with the miR-29c mimic. Consistent with these results, transfection with c-Jun shRNA reversed the inhibitory effects of the miR-29c inhibitor on cell apoptosis (Fig. 9).

Discussion

Pan *et al* (11) provided the first evidence of the expression of a unique set of miRNAs in the endometrium and endometrial cells, suggesting that a substantial number of miRNAs are aberrantly or differentially expressed in the ectopic endometrium. Ohlsson Teague *et al* (12) profiled miRNAs in paired samples of eutopic and peritoneal ectopic endometrial tissue from patients with endometriosis using miRNA microarray analysis. Of the miRNAs evaluated, 22 miRNAs showed a differential expression in the paired eutopic vs. the ectopic endometrium samples. Both the studies of Pan *et al* (11) and Ohlsson Teague *et al* (12) revealed that miR-29c was differentially expressed in the eutopic and ectopic endometrium. miR-29c belongs to the miR-29 family, and it has been suggested to be downregulated in certain types of cancer (13-16). In the present study, the expression levels

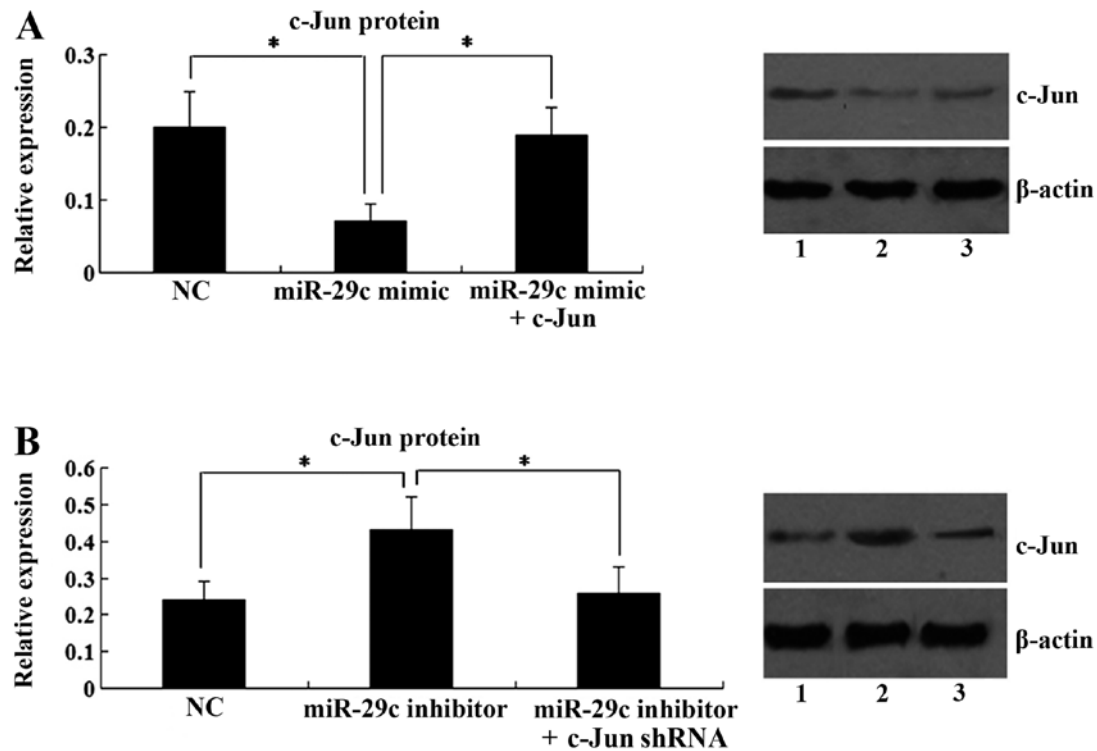


Figure 6. Protein expression of c-Jun following transfection with the miR-29c mimic/inhibitor and c-Jun overexpression/shRNA plasmid in CRL-7566 cells. (A) c-Jun overexpression plasmid reversed the decrease in c-Jun protein expression in miR-29c mimic-transfected cells. Lane 1, NC; lane 2, miR-29c mimic; lane 3, miR-29c mimic + c-Jun. (B) c-Jun shRNA plasmid reversed the increase in c-Jun protein expression in miR-29c inhibitor-transfected cells. Lane 1, NC; lane 2, miR-29c inhibitor; lane 3, miR-29c inhibitor + c-Jun shRNA. * $P < 0.05$. NC, negative control.

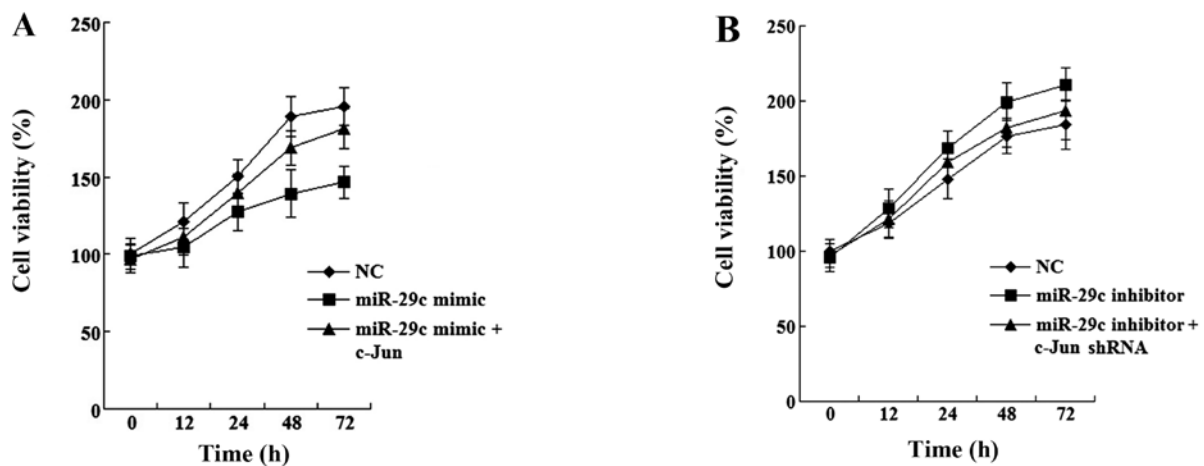


Figure 7. c-Jun reverses the miR-29c-induced suppression of cell proliferation. (A) Cell viability following transfection with the miR-29c mimic and the c-Jun overexpression vector. (B) Cell viability following transfection with the miR-29c inhibitor and the c-Jun shRNA. NC, negative control.

of the miR-29 family in the endometrium samples from women without endometriosis, as well as in paired ectopic and eutopic endometrial tissue samples from patients with endometriosis were examined by RT-qPCR. It was revealed that miR-29c was differentially expressed in the paired eutopic and ectopic endometrium samples. These findings indicate that miR-29c may be associated with the progression of endometriosis.

To determine the potential role of miR-29c in endometrial cells, miR-29c mimic and miR-29c inhibitor were transfected into CRL-7566 cells. MTT assay revealed that the cells trans-

fected with the miR-29c mimic proliferated at a significantly lower rate, and transfection with miR-29c inhibitor had the opposite effect. Endometriotic cells have an invasive potential which may contribute to the pathogenesis of endometriosis (10). In the present study, we found that transfection with miR-29c mimic suppressed cell invasion ability, while transfection with the miR-29c inhibitor enhanced cell invasion ability. FCM was used to examine the effects of miR-29c on cell apoptosis. As expected, transfection with miR-29c mimic increased the cell apoptotic rate, while transfection with the miR-29c inhibitor

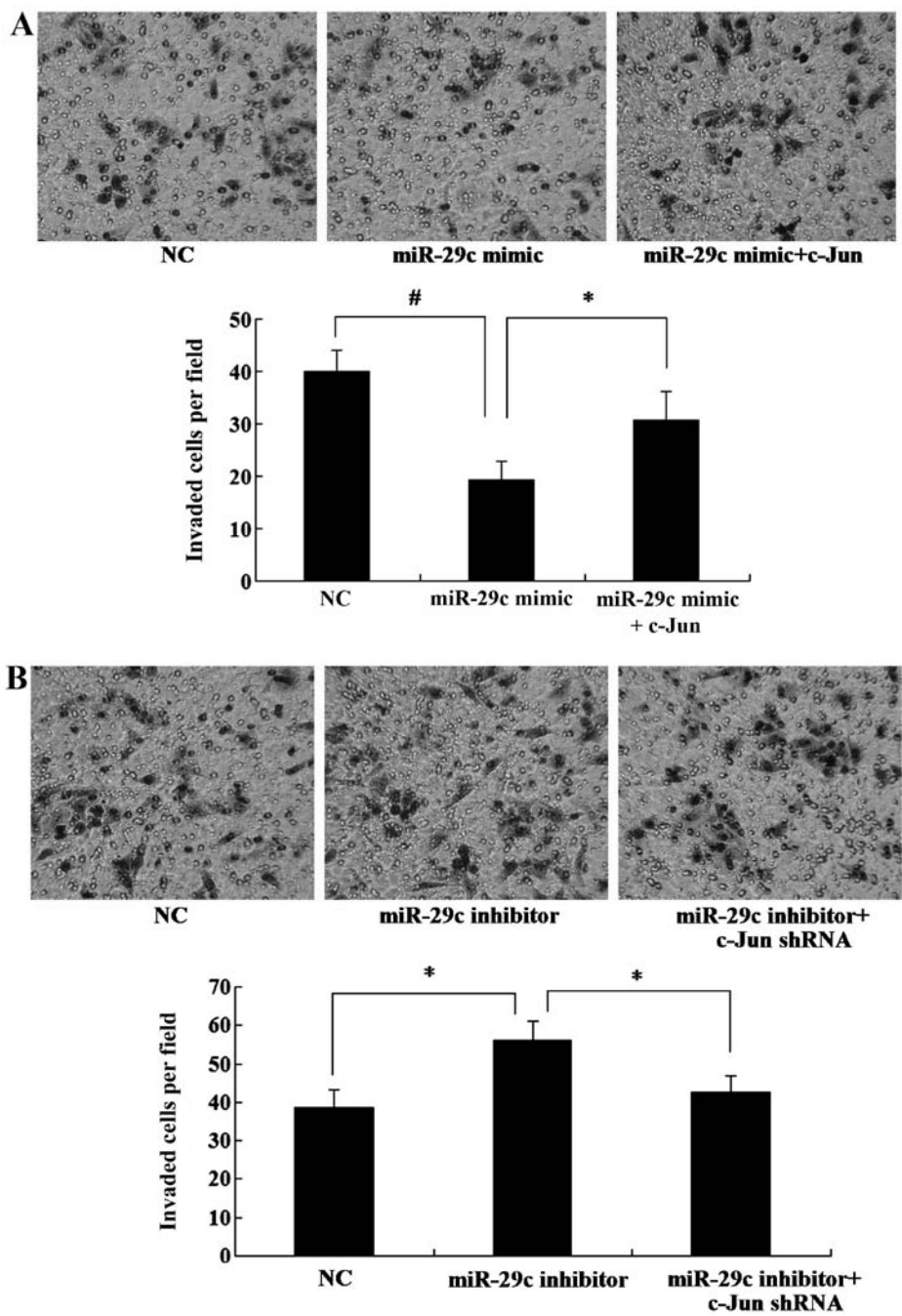


Figure 8. c-Jun reverses the miR-29c-induced suppression of cell invasion. (A) Number of invaded cells following transfection with the miR-29c mimic and the c-Jun overexpression vector. (B) Number of invaded cells following transfection with the miR-29c inhibitor and the shRNA-c-Jun. * $P<0.05$ and $^{\#}P<0.01$. NC, negative control.

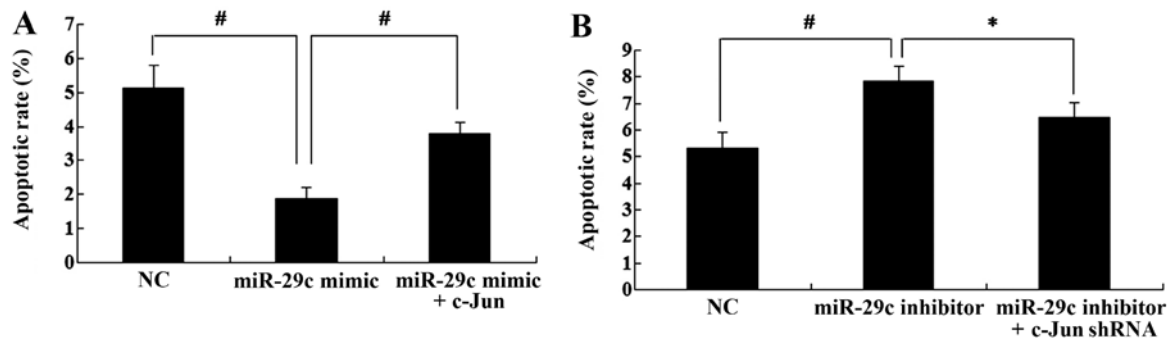


Figure 9. c-Jun reverses the miR-29c-mediated induction of cell apoptosis. (A) Cell apoptotic rate following transfection with the miR-29c mimic and the c-Jun overexpression vector. (B) Cell apoptotic rate following transfection with the miR-29c inhibitor and the shRNA-c-Jun. * $P<0.05$ and $^{\#}P<0.01$. NC, negative control.

had the opposite effect. These data indicate that miR-29c potentially plays a negative role in the development of endometriotic lesions. miR-29c may exert its effects on endometrial cells by suppressing cell proliferation and invasion, as well as by promoting cell apoptosis.

Studies on the differences in gene expression between the ectopic and eutopic endometrium (17-22) have found that many genes related to cell adhesion, cell migration, cell proliferation, immune regulation and inflammation are differentially expressed in ectopic vs. eutopic endometrial tissue. In the study by Ohlsson Teague *et al* (12), it was suggested that c-Jun mRNA expression was significantly increased in endometriosis. c-Jun is a transcription factor which belongs to the activating protein 1 family (23). Genetic studies have demonstrated that c-Jun regulates a variety of genes important for diverse cellular functions, including cell differentiation, apoptosis, proliferation, migration and invasion (24-26). The overexpression of c-Jun has been detected in various types of tumors (27,28). In the present study, it was revealed that c-Jun was also differentially expressed in the ectopic vs. eutopic endometrial tissue as determined by western blot analysis. This result is consistent with the results of a previous study demonstrating that c-Jun is associated with endometriosis (12).

We predicted the targets of miR-29c by miRanda and focused on c-Jun. Luciferase reporter assay and western blot analysis revealed that miR-29c directly binds to the c-Jun gene and inhibits the protein expression of c-Jun. These results indicate a negative role of miR-29c in the regulation of c-Jun expression and provide support for our hypothesis that miR-29c suppresses the progression of endometriosis possibly through the downregulation of c-Jun. In addition, c-Jun overexpression plasmid and shRNA plasmid were transfected into the CRL-7566 cells. The results revealed that the overexpression of c-Jun reversed the inhibitory effects of the miR-29c mimic on cell proliferation and invasion, and the overexpression of c-Jun reversed the increase in the cell apoptotic rate induced by the miR-29c mimic. In addition, c-Jun shRNA reversed the effects of the miR-29c inhibitor on cell proliferation, invasion and apoptosis. Thus, our results suggest that c-Jun plays an important role in the regulation of the proliferation, invasion and apoptosis of endometrial cells by miR-29c.

In conclusion, we identified miR-29c as a suppressor of endometriosis, and that it is differentially expressed in paired ectopic and eutopic endometrial tissues. miR-29c exerts its effects on endometrial cell proliferation, apoptosis and invasion by inhibiting the expression of c-Jun. This study may provide a novel potential therapeutic target for the treatment of endometriosis.

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