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MicroRNA-2861 targets STAT3 and MMP2 to regulate the proliferation and apoptosis of ectopic endometrial cells in endometriosis

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This study aimed to elucidate the roles and regulatory mechanism of miR-2861 in the development of endometriosis. The expression of miR-2861 in endometriotic tissues and eutopic endometrial tissues was determined. Ectopic endometrial cells were transfected with miR-2861 mimic, miR-2861 inhibitor and their corresponding controls. The effects of miR-2861 overexpression and inhibition on cell proliferation and apoptosis were then investigated. In addition, regulatory relationship between miR-2681 and signal transducer and activator of transcription 3 (STAT3) or matrix metalloproteinase 2 (MMP2) was explored, as well as between STAT3 and MMP2. Compared with eutopic endometrial tissues, miR-2861 was significantly downregulated in endometriotic tissues. Overexpression of miR-2861 markedly inhibited ectopic endometrial cell proliferation and induced apoptosis. Additionally, STAT3 and MMP2 were confirmed as the targets of miR-2861, and the effects of knockdown of STAT3 or MMP2on regulating cell proliferation and apoptosis were opposite with inhibition of miR-2861. Besides, STAT3 could enhance the expression of MMP2 in ectopic endometrial cells. Our study reveals that miR-2861 was lowly expressed in endometriotic tissues, and downregulation of miR-2861 may promote proliferation and inhibit apoptosis of ectopic endometrial cells in endometriosis *via* upregulation of STAT3 and MMP2. miR-2861 may be a potential biomarker or therapeutic target for endometriosis.

1. Introduction

Endometriosis is an estrogen-dependent inflammatory disease, which is characterized by endometrial-type mucosa outside the uterine cavity (Iwabe and Harada 2014; Vercellini et al. 2014). The prevalence of endometriotic disease is approximately 5% and reaches it's peak between the ages of 25 and 35 years (Giudice et al. 2012; Viganò et al. 2004). Endometriosis is always accompanied by several pain symptoms, including dysmenorrhoea, low back pain, dyspareunia, dysuria and dyschezia (Tokushige 2014). Current treatments of endometriosis are surgery and ovarian suppressive agents, including GnRh agonists, progestins, oral contraceptives, and androgenic agents; however, hormonal treatments are associated with side effects, such as delayed conception (Elnashar 2015). Seriously, endometriosis is found responsible for a 50 % increase in the risk of epithelial ovarian cancer (Vercellini et al. 2014). Therefore, it is still necessary to look for effective treatments for endometriosis.

MicroRNAs (miRNAs) are small, non-coding RNAs with lengths of 20-25 nucleotides and have been reported to be implicated in multiple physiologic and pathologic processes *via* modulating the gene expression of specific targets (Kloosterman and Plasterk 2006; O'Connell et al. 2010). Dysregulation of miRNAs is proposed to be key regulators in various cellular processes that occur in endometriosis (Braza-Boïls et al. 2013, 2014; Laudanski et al. 2013; Nothnick 2017). In addition, miRNAs have emerged as potential biomarkers for the diagnosis of endometriosis (Cho et al. 2015; Cosar et al. 2016). Recently, miR-2861 has been found to play a role in regulating the development of papillary thyroid carcinoma (Wang et al. 2013). It is reported that miR-2861 plays a tumor suppressor role in human papillomavirus virus 16 E6-induced cervical cancer *via* targeting the EGFR/AKT2/CCND1 pathway (Xu et al. 2016). miR-2861 is also identified as a non-invasive biomarker for detecting cervical cancer (Zhang et al. 2015). Despite these, there were limited reports on the roles of miR-2861 in endometriosis.

In this study, we analyzed the expression of miR-2861 in endometriotic tissues and investigated the effects of miR-2861 dysregulation on cell proliferation and apoptosis. In addition, the regulatory relationship between miR-2681 and signal transducer and activator of transcription 3 (STAT3) or matrix metalloproteinase 2 (MMP2) was explored to further elucidate the mechanism of miR-2681 on endometriosis. Our findings will provide a theoretical basis for a better understanding of the molecular mechanism of endometriosis.

2. Investigations and results

2.1. miR-2861 was downregulated in endometriotic tissues

To investigate whether miR-2861 was a key regulator in the development of endometriosis, the expression of miR-2861 was measured by qRT-PCR. The results show that miR-2861 was downregulated in endometriotic tissues compared with eutopic endometrial tissues ($P<0.05$, Fig. 1A), indicating that miR-2861 may play a key role in endometriosis development.

2.2. miR-2861 was successfully overexpressed and suppressed in ectopic endometrial cells

To explore the role of miR-2861 in endometriosis development, ectopic endometrial cells were transfected with miR-2861 mimic, miR-2861 inhibitor and their corresponding controls. In comparison with their corresponding controls, cell viability of miR-2861 mimic transfected cells was significantly decreased with the increase of

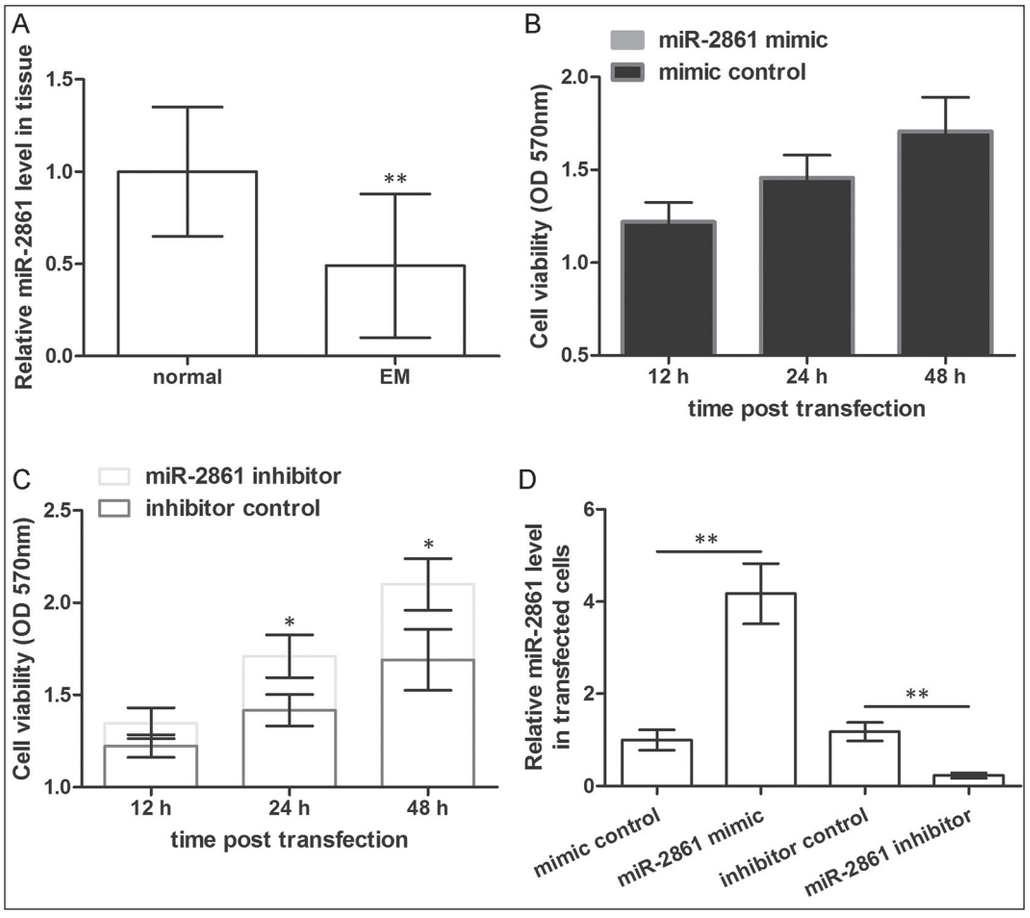

Fig. 1: miR-2861 was downregulated in endometriotic tissues and miR-2861 was successfully overexpressed and suppressed in ectopic endometrial cells. A: The expression level of miR-2861 in endometriotic tissues and eutopic endometrial tissues. B: MTT showed the cell viability of miR-2861 mimic and mimic control transfected cells. C: MTT showed the cell viability of miR-2861 inhibitor and inhibitor control transfected cells. D: The expression of miR-2861 in different transfected groups. *p < 0.05; **p < 0.01.

transfection time (P<0.05, Fig. 1B), while cell viability of miR-2861 inhibitor transfected cells was gradually promoted (P<0.05, Fig. 1C). Moreover, the expression of miR-2861 was markedly increased after transfection with miR-2861 mimic, while obvious decreased after transfection with miR-2861 inhibitor (P<0.05, Fig. 1D). These data confirmed that miR-2861 was successfully overexpressed and suppressed in ectopic endometrial cells.

2.3. Inhibition of miR-2861 promoted proliferation and inhibited apoptosis of ectopic endometrial cells

As shown in Figs. 2A and 2B, the BrdU-positive cells in miR-2861 mimic group were significantly decreased compared with the mimic control group, while the BrdU-positive cells in miR-2861 inhibitor group were markedly increased compared with inhibitor control group (P<0.05). Moreover, in comparison with their corresponding control groups, the apoptotic cells were significantly increased in miR-2861 mimic group, while markedly decreased in miR-2861 inhibitor group (P<0.05, Figure 2C and 2D). Besides, the expression ratio of BAX/BCL2 was enhanced after transfection with miR-2861 mimic, while obviously decreased after transfection with miR-2861 inhibitor (P<0.05, Figs. 2E and 2F).

2.4. STAT3 and MMP2 were directly targets of miR-2861

The potential targets of miR-2861 were also predicted using the online database TargetScanHuman 7.1 (http://www.targetscan.org/vert_71/), including STAT3 and MMP2 (Fig. 3A). To confirm the predicted results, luciferase reporter assay was carried out and the results showed that miR-2861 mimic significantly inhibited the luciferase activity of STAT3 or MMP2 3'UTR-wt (P < 0.05, Figs. 3B and 3C), but could not target STAT3 or MMP2 3'UTR-mut. Compared with their corresponding control groups, the expression levels of STAT3 and MMP2 were significantly decreased in miR-2861 mimic group, while markedly increased in miR-2861 inhibitor group (P < 0.05, Figs. 3D and 3E). Besides, the expression levels of STAT3 and MMP2 in endometriotic tissues were significantly higher than that in eutopic endometrial tissues (P < 0.05, Figs. 3F and 3G). These findings confirmed that STAT3 and MMP2 were directly targets of miR-2861.

2.5. Knockdown of STAT3 and MMP2 inhibited proliferation and induced apoptosis of ectopic endometrial cells

To further confirm whether STAT3 and MMP2 were involved in the regulatory roles of miR-2861 in ectopic endometrial cells, the expression levels of STAT3 and MMP2 were knocked down by si-STAT3 and si-MMP2 (P < 0.05, Fig. 4A), respectively. Moreover, the BrdU-positive cells in si-STAT3 and si-MMP2 groups were all significantly decreased compared with scramble control group (P<0.05, Fig. 4B). However, the apoptotic cells in si-STAT3 and si-MMP2 groups were significantly increased compared with scramble control group (P<0.05, Fig. 4C), as well as expression ratio of BAX/BCL2 (P<0.05, Fig. 4D).

2.6. STAT3 enhanced the expression of MMP2 in ectopic endometrial cells

MMP2 expression was also significantly downregulated after knockdown of STAT3 (P < 0.05, Fig. 4A), implying that STAT3 might inhibit the expression of MMP2. Additionally, the effects of STAT3 knockdown on the proliferation and apoptosis of ectopic endometrial cells were more obvious than the effects of MMP2 knockdown (P < 0.05, Fig. 4A-D). To verify the enhanced effects

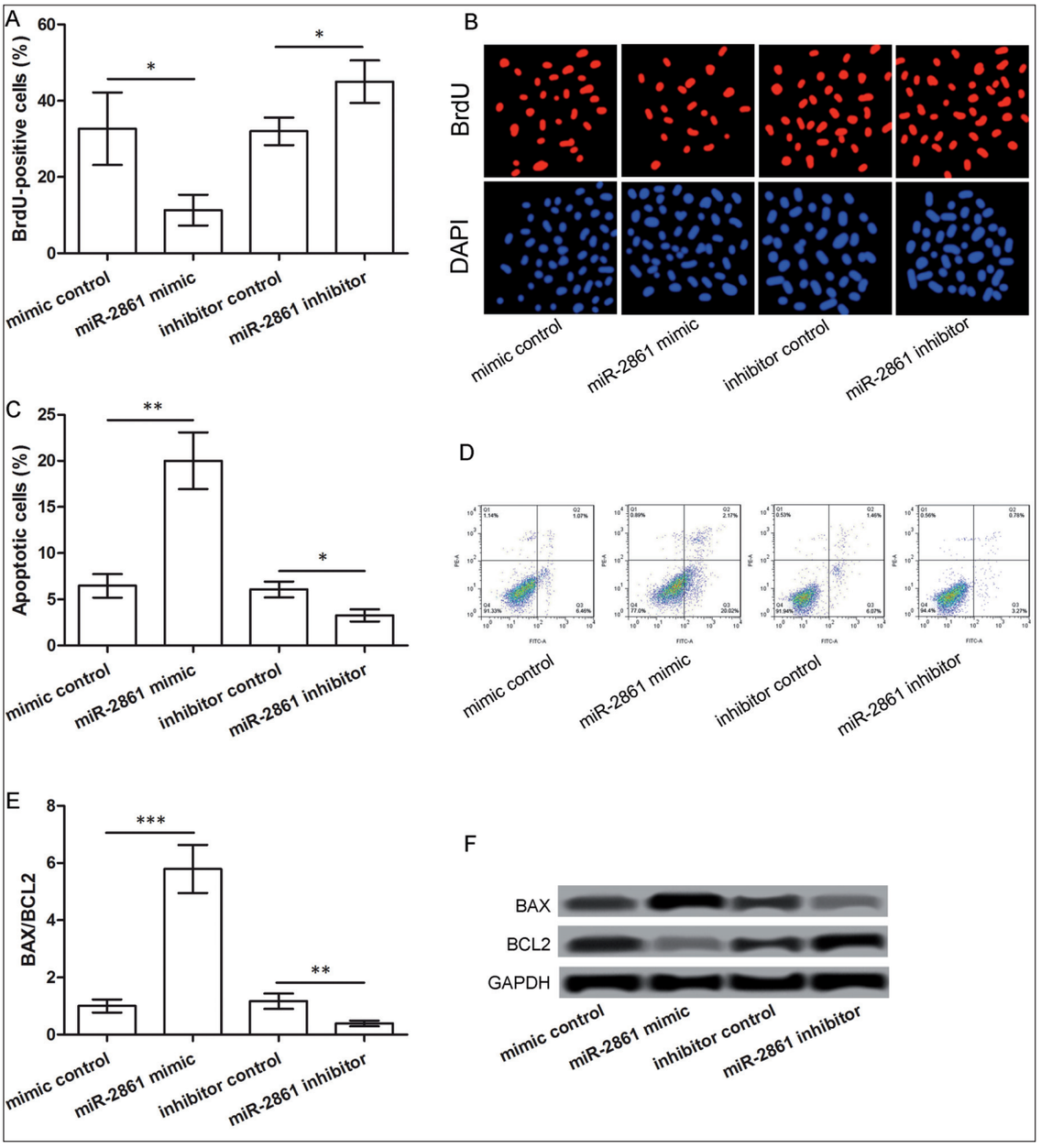

Fig. 2: Inhibition of miR-2861 promoted proliferation and inhibited apoptosis of ectopic endometrial cells. A and B: The BrdU-positive cells in different transfected groups. C and D: Flow cytometry showed the apoptotic cells in different transfected groups. E and F: Western blot showed the expression levels of BAX and BCL2 in different transfected groups. *$p < 0.05$; **$p < 0.01$, *** $p < 0.001$.

of STAT3 on MMP2 expression, ectopic endometrial cells were transfected with pcDNA-STAT3, pcDNA-MMP2 and blank vector. The results show that STAT3 and MMP2 were all upregulated in ectopic endometrial cells after transfection with pcDNA-STAT3, while only MMP2 was upregulated in ectopic endometrial cells after transfection with pcDNA-MMP2 ($P < 0.05$, Figs. 5A and 5B). These data imply that STAT3 may promote MMP2 expression in ectopic endometrial cells.

3. Discussion

Endometriosis is a common chronic disease in gynecology, yet its pathogenesis remains largely unknown (Shi et al. 2014). In this study, we found that miR-2861 was significantly downregulated in endometriotic tissues compared with eutopic endometrial tissues. Overexpression of miR-2861 markedly inhibited ectopic endometrial cell proliferation and induced apoptosis, imply the inhibitory effects on endometriosis. Additionally, STAT3 and MMP2 were confirmed as the targets of miR-2861, and knockdown of STAT3 or MMP2 had opposite effects with inhibition of miR-2861 on regulating cell proliferation and apoptosis. Besides, STAT3 may promote the expression of MMP2 in ectopic endometrial cells, thus to affect the effects of MMP2 on endometriosis. These findings highlight the biological actions of miR-2861 in endometriosis and merit further discussion.

miRNAs play a vital role in the regulation of the endometriosis development, including miR-199a-5p (Hsu et al. 2014) and miR-451(Graham et al. 2015). This study found the downregulation of miR-2861 in endometriotic tissues, however, few studies have investigated the roles of miR-2861 in endometriosis. It has been reported that miR-2861 can inhibit cell proliferation and promote apoptosis in cervical cancer (Xu et al. 2016), indicating that miR-2861 may act as a tumor suppressor in cervical cancer. Similarly, we found that miR-2861 markedly inhibited ectopic endometrial cell proliferation and induced apoptosis, imply the inhibitory effects on endometriosis.

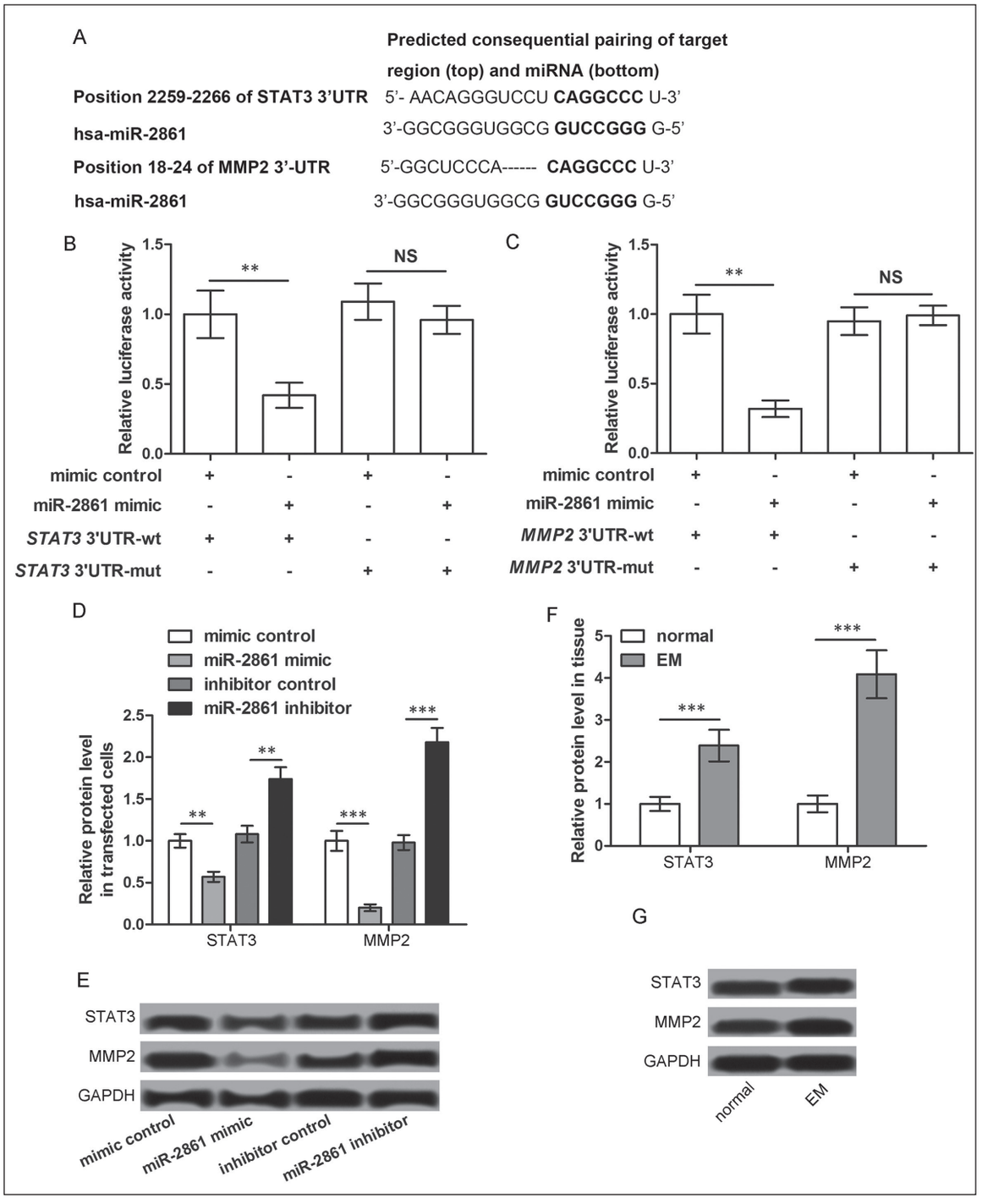

Fig. 3: STAT3 and MMP2 were directly targets of miR-2861. A: The predicted target sequences of miR-2861 and STAT3 or MMP2 using the online database TargetScanHuman 7.1. B and C: Luciferase reporter assay showed the luciferase activity of STAT3 or MMP2 3'UTR-wt and STAT3 or MMP2 3'UTR-mut after cotransfection with miR-2861 mimic or mimic control. D and E: Western blot showed the expression levels of STAT3 and MMP2 in different transfected groups. F and G: Western blot showed the expression levels of STAT3 and MMP2 in endometriotic tissues and eutopic endometrial tissues. **$p < 0.01$, *** $p < 0.001$.

Furthermore, this study confirmed the target genes of miR-2861 were STAT3 and MMP2. In women with endometriosis, STAT3 activation may be central to the inflammatory phenotype of eutopic endometrium (Yoo et al. 2016). It has also been reported that aberrant activation of STAT3 signaling plays a key role in the pathogenesis of endometriosis (Kim et al. 2015). Moreover, the interaction between endometrial stromal cells and peritoneal M2 macrophages contributes to endometriosis development by activation of STAT3 (Itoh et al. 2014). Oncostatin M is found to promote tumor invasion and angiogenesis in endometrial cancer through activating of STAT3 (Zhu et al. 2015). In this study, knockdown of STAT3 inhibited proliferation and induced apoptosis of ectopic endometrial cells, which was opposite with the effects of miR-2861 inhibition. Therefore, we speculate that downregulation of miR-2861 may promote endometriosis development *via* activation of STAT3.

On the other hand, MMP2 is found to be related with the changes in steroid hormones, thus resulting in the formation of endometriosis (Huang et al. 2004). Malvezzi et al. (2013) also demonstrated that the severity of advanced pelvic endometriosis was related to higher serum MMP-2 levels (Malvezzi et al. 2013). A miR-520 mirSNP at the MMP2 gene is confirmed to affect the susceptibility to endometriosis in Chinese women (Tsai et al.

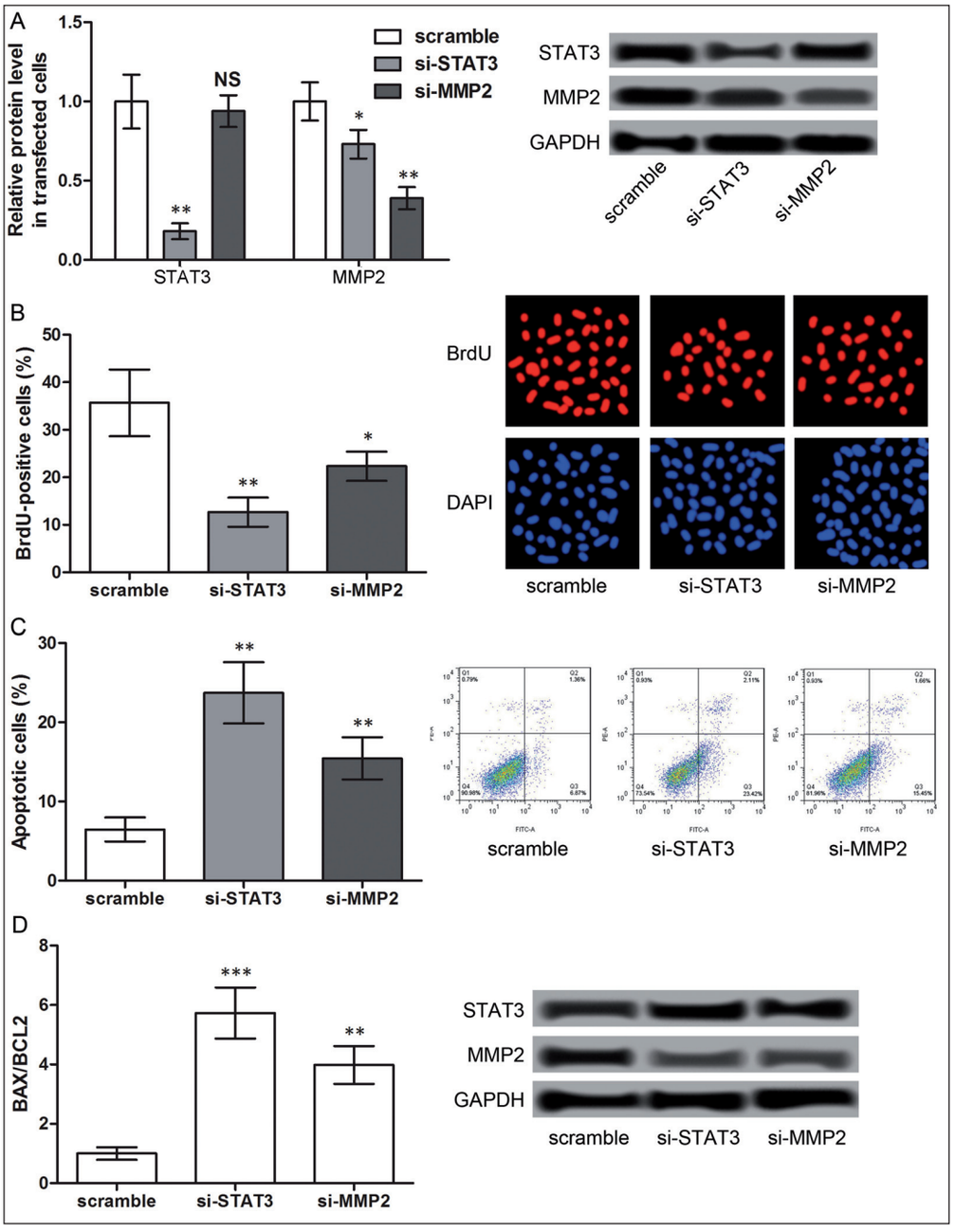

Fig. 4: Knockdown of STAT3 and MMP2 inhibited proliferation and induced apoptosis of ectopic endometrial cells. A: The expression levels of STAT3 and MMP2 after transfection with si-STAT3, si-MMP2 and scramble control. B: The BrdU-positive cells in different transfected groups. C: Flow cytometry showed the apoptotic cells in different transfected groups. D: Western blot showed the expression levels of BAX and BCL2 in different transfected groups. *p < 0.05; **p < 0.01, *** p < 0.001.

2013). In our study, the effects of knockdown of STAT3 on proliferation and apoptosis of ectopic endometrial were also opposite with the effects of miR-2861 inhibition. These data indicate that downregulation of miR-2861 may promote endometriosis development *via* regulating MMP2. Notably, our results also showed that MMP2 expression was significantly downregulated after knockdown of STAT3, and STAT3 and MMP2 were all upregulated in ectopic endometrial cells after transfection with pcDNA-STAT3. The effects of STAT3 knockdown on the proliferation and apoptosis of ectopic endometrial cells were more obvious than the effects of MMP2 knockdown. Leptin is reported to promote the migration and invasion of endometriotic cells by upregulation of MMP-2 *via* the JAK2/STAT3 signaling pathway (Ahn et al. 2015). Taken together, we hypothesize that miR-2861 may promote endometriosis development *via* regulating MMP2 through activation of STAT3 signaling.

In conclusion, our study reveals that miR-2861 is lowly expressed in endometriotic tissues and downregulation of miR-2861 may promote endometriosis development *via* upregulation of STAT3 and MMP2. These findings suggest that miR-2861 may be a potential biomarker or therapeutic target for endometriosis. Further experimental studies are still needed for the verification of our findings.

4. Experimental

4.1. Tissue sampling

Under a protocol approved by local committee, this study was conducted. All participants provided written informed consent for participation. A total of 19 patients who suffered from endometriosis (mean age 39.3 years; range 34-43 years) were enrolled into this study, and their eutopic endometrial and endometriotic tissues were obtained by laparoscopy. Six months before the surgical operation, patients had not received any hormonal drugs, such as GnRH analog or other. All the samples were confirmed histologically. Eutopic endometrium acquired from 12 disease-free women was considered as healthy controls.

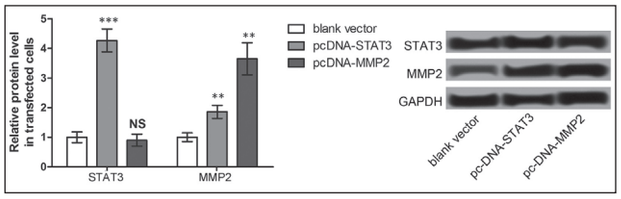

Fig. 5: The expression levels of STAT3 and MMP2 in ectopic endometrial cells which were transfected with pcDNA-STAT3, pcDNA-MMP2 and blank vector. **p < 0.01, *** p < 0.001.

4.2. Cell isolation and culture

The endometriotic tissues were cut to little pieces and then incubated with enzymatic dissociation solution for 10 min. After discarding the dissociation solution, the isolated ectopic endometrial cells were resuspended with compete Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal calf serum in a 95 % humidified incubator 37 °C, 5 % CO_2.

4.3. Cell transfection

The cells were transfected with miR-29c mimic, miR-29c inhibitor, and their corresponding controls, mutant and wild-type STAT3 or MMP2 3'UTR, siRNA for STAT3 or MMP2, and the overexpression plasmid of STAT3 or MMP2 using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions and then incubated at 37 °C for 24 h.

4.4. MTT assay

Cell viability was evaluated with a MTT assay. The cells were seeded into 96-well culture plates at a density of $1x10^4$ cells/well. At 12, 24 and 48 after transfection, to each well 10 µl MTT solution (0.5 mg/ml; Beyotime, Shanghai, China) was added and incubation was continued for 4 h. Afterwards, DMSO (Sigma, St. Louis, MO, USA) was added to dissolve the MTT formazan. Cell viability of each well was read at 570 nm on a Multiskan Ascent 354 microplate reader (Thermo Labsystems, Waltham, MA, USA).

4.5. BrdU cell proliferation assay

Cells were harvested, washed with PBS, fixed with 4 % paraformaldehyde for 15 min, permeabilized with 0.1 % Triton/PBS, denatured with 0.1 N HCl, washed in PBST (1# PBS Tween-20), blocked with 5 % BSA/PBST, and then incubated with the anti-BrdU antibody (1:100, Sigma, USA). After washing with PBST for three times, cells were incubated with the appropriate Cy-2- or Cy-3–conjugated secondary antibody (Jackson Immunoresearch) away from the light for 45 min. Cells were counterstained with DAPI, mounted with antifade (Vectastain), and visualized by fluorescence microscopy.

4.6. Flow cytometry analysis

Cell apoptosis was assessed by flow cytometry. Briefly, cells were resuspended in binding buffer and then double stained with Annexin V and propidium iodide (PI) (Kaiji Biological Inc., Nanjing, China) according to the manufacturer's instructions of a FITC Annexin V Apoptosis Detection Kit (BD Biosciences, Shanghai, China). Apoptotic cells were then detected and analyzed by flow cytometer (BD FACSAria; BD Biosciences, Franklin Lakes, NJ, USA).

4.7. Luciferase report assay

The 3'-UTRs of human *STAT3* and *MMP2* and their corresponding mutated 3'-UTRs were amplified by PCR. Cells (2×10^4) were seeded into a 48-well plate. Sequences from the miR-2861 target site in the 3'-UTR of *STAT3* and *MMP2* and their mutant variants were cloned into the psiCHECK-2 vector (Promega), which were then contransfected with miR-2861 mimic or mimic control into cells. 48 h after transfection, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase activity was used as the internal control.

4.8. Quantitative real-time PCR (qRT-PCR)

Following the manufacturer's instructions, the miRNAs was extracted by a miRNeasy Mini kit (Qiagen, Hilden, Germany). Reverse transcription into cDNA was then conducted with a RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania). To detect the expression of miR-2861, qRT-PCR was performed using a SYBRGreen PCR kit (both from Applied Biosystems, Foster City, CA, USA) by means of a 7900 Real-Time PCR System. After normalization with the expression of U6, the relative expression of miR-2861was analyzed using the comparative ($2^{-\Delta\Delta Ct}$) method.

4.9. Western blot

Total protein was extracted from cells using a total protein extraction kit (Kaiji Biological, Inc.), which concentration was then measured with the BCA kit (Beyotime). The equal amount of samples was then separated in 10 % SDS-PAGE and blotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocked in 5 % non-fat milk, the membranes were incubated with primary antibodies, including mouse monoclonal to STAT3, MMP2 and GAPDH (1:1000, Abcam, Cambridge, MA, USA) overnight at 4 °C, followed by incubation with secondary antibody conjugated with horseradish peroxidase (1:5000; BA1050; Boster, Wuhan, Hubei, China) for 2 h. GAPDH was used as a control. The ECL detection kit (Pierce, Rockford, IL, USA) was used to visualize the protein blots, and the densitometry of proteins was then analyzed with Quantity One software (Bio-Rad, CA, USA).

4.10. Statistical analysis

All experiments were performed three times at least, and the related data were shown as the mean±SD. Data were analyzed with Student's t-test or one-way analysis of variance (ANOVA) in Statistical Package for the Social Sciences (SPSS) software version 11.5 (IBM, IL, USA). Statistically significant differences were obtained at $P<0.05$.

Conflicts of interest: None reported.

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