European Review for Medical and Pharmacological Sciences 2019; 23: 4073-4080

The modulation of endometriosis by lncRNA MALAT1 via NF-κB/iNOS

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Abstract. – OBJECTIVE: Endometriosis (Ems) is one benign disorder that frequently leads to chronic pelvic pain, dysmenorrhea or even infertility. Featured as the ectopic growth of active endometrial tissues on tissues beyond the muscular layer, Ems has complicated pathogenesis mechanisms that are not fully illustrated. Long non-coding (lnc) RNA MALAT1 participates in various biological activities, including cell growth, proliferation, apoptosis, organogenesis, inflammation, and tumors. However, its expression and function in Ems are not clear yet.

PATIENTS AND METHODS: Real-time PCR measured differential expression of MALAT1 in normal and ectopic endometrial tissues. Cultured endometrial cells were transfected with siRNA for MALAT1, whose expression was measured by real-time PCR. MTT assay measured endometrial cell proliferation, whose invasion was measured by transwell assay. Western blot tested the expression and NF-κB/iNOS, and MMP9 proteins.

RESULTS: LncRNA MALAT1 showed upregulation in ectopic endometrial tissues ($p<0.05$ comparing to control group). Transfection of MALAT1 siRNA suppressed its expression in endometrial cells, inhibited cell proliferation or invasion, enhanced caspase 3 activity, decreased NF-κB/iNOS or MMP-9 expression ($p<0.05$ comparing to control group).

CONCLUSIONS: LncRNA MALAT1 can facilitate endometrial cell apoptosis and modulate MMP-9 expression via NF-κB/iNOS pathway, thus, mediating Ems pathogenesis.

Key Words:
Endometriosis, LncRNA MALAT1, Cell proliferation, Cell invasion, NF-κB/iNOS.

Introduction

As one common and popular disease in gynecology, endometriosis (Ems) is formed by the ectopic implantation of active endometrial cells into the non-endometrium site. Although belonging to the benign lesion, Ems severely affects health and lifestyle of women[1,2]. Major pathological changes of Ems include the entry of endometrial cells into the pelvic cavity or muscular layer of the uterus via fallopian tube, causing cyclic hemorrhage of ectopic endometrial cells and peripheral fibrosis, leading to ectopic lesion, dysmenorrhea, chronic pelvic pain, abnormal menstruation or even infertility[3,4]. Ems is frequently accompanied with tumor-like infiltrative growth patterns, with certain recurrent and malignant transformation rate[5]. Ems can occur in the ovary, rectal-uterus junction or bladder-uterus junction. Having an increasing rate of incidence, Ems causes severe pain in patients and, thus, it has received a considerable amount of attention in clinics[6,7]. Currently, treatment for Ems includes medication and surgery. Drug treatment has issues of adverse effects and high recurrence, whilst radical surgery is usually incompatible on unborn women. The conservative treatment is also challenged by high recurrent rate[8,9]. Ems has a complicated pathogenic mechanism, including immune deficiency, genetic factors, iatrogenic endometriosis, and implantation hypothesis, but still having no comprehensive illustrations[10,11]. Therefore, the identification of related molecular target can benefit the illustration of Ems pathogenic mechanism and improve treatment efficiency.

Long non-coding RNA (lncRNA) is one group of recently identified non-coding RNA transcript with more than 200 nt length[12]. Such non-coding transcript is abundantly found in human genome. As not participating in protein coding, they were initially regarded as the transcription "noise". However, further investigations found that they can modulate gene expression at epigenetic, transcriptional regulation and post-transcriptional regulation levels[13,14]. lncRNA also plays important roles in the onset and progression of multiple diseases including tumors[15]. LncRNA MALAT1

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is one lncRNA molecule widely studied and has been demonstrated to participate in various pathology-physiological processes[16]. However, the role of lncRNA MALAT1 in EMS or its function has not been clearly illustrated.

Patients and Methods

Research Subjects and Sample Collection

A total of 15 Ems patients who were diagnosed by pathology examination in Yantai Yuhuangding Hospital (Yantai, Shandong, China) between April 2017 and May 2018 were recruited. Patients aged between 29 and 46 years (average age = 41.2 ±5.8 years). Inclusive and exclusive criteria: All patients were diagnosed firstly and samples were obtained during the surgery. Patients have not received medication, radio- or chemotherapy, or hormonal replacement previously. Patients had no intrauterine device implantation. Those patients complicated with other reproductive disorders were excluded, along with those having severe organ failure, malignant tumors or complications[10]. Another cohort of 7 patients who have been collected for endometrial tissues due to uterine prolapse or hysteromyoma was used as the control group, with age ranging between 31 and 45 years (average age = 39.6±7.3 years). No statistical significance has been found in clinical information between two groups. Thus, they were comparable. Endometrial tissues collected during the surgery were partially kept in DMEM (Dulbecco's Modified Eagle's Medium), and some tissues were frozen at -80°C for further use.

Ethics Statement

All research subjects have signed informed consents. This study has been approved by The First Affiliated Hospital of Fujian Medical University (Fujian, China).

Major Equipment and Reagents

DMEM medium, fetal bovine serum (FBS), and penicillin-streptomycin were purchased from HyClone (GE Healthcare Life Sciences, HyClone Laboratories, South Logan, UT, USA). DMSO and MTT powder were purchased from Gibco (Grand Island, NY, USA). Trypsin-EDTA digestion buffer was purchased from Sigma-Aldrich (St. Louis, MO, USA). Caspase 3 activity assay kit was purchased from Pall Life Science (St. Louis, MO, USA). Transwell chamber was purchased from Corning (Shanghai, China). PVDF membrane was purchased from Pall Life Science (Port Washington, NY, USA). EDTA was purchased from Hyclone. Western blot reagents were purchased from Beyotime Biotech (Jiangsu, China). ECL reagent was purchased from Amersham Biosciences (Piscataway, NJ, USA). Rabbit anti-human NF-κB and anti-iNOS monoclonal antibody, rabbit anti-human MMP-9 monoclonal antibody, and mouse anti-rabbit horseradish peroxidase (HRP) conjugated IgG secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). TaqMan microRNA reverse transcription kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). lncRNA MALAT1 siRNA and negative control (NC) sequences were synthesized by Gimma Gene (Shanghai, China). RNA extraction kit and reverse transcription kit were purchased from AXYGEN co. (Union City, CA, USA). Other common reagents were purchased from Axygen (Corning, NY, USA). Other common reagents were purchased from Sangon Biotech (Shenzhen, China). LabSystem Version 1.3.1 microplate reader was purchased from Bio-Rad (Hercules, CA, USA). ABI 7700 Fast fluorescent quantitative PCR cycler was purchased from ABI (Vernon, CA, USA). Ultrapure workstation was purchased from Sutai Purification equipment (Suzhou, China). Biosafer 1000 ultrasonic rupture was purchased from Saifei (Shanghai, China). Thermo Scientific Forma CO_2 incubator was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Primary Culture of In Situ Endometrial Cells and Grouping

Endometrial tissues were rinsed in sterile PBS for 2-3 times and were cut into 0.5-1 cm^3 cubes. Tissues were digested by 0.25% trypsin, 0.1% collagenase IV and 0.1% hyaluronidase at 37°C for 60 min until no obvious tissue cubes. The tissue lysate was centrifuged at 1000 rpm or 5 min to discard the supernatant. 1 ml freshly prepared DMEM medium was added for re-suspension for two times of rinsing. 1 ml fresh DMEM medium containing 10% FBS and 100 U/ml penicillin plus 100 μg/ml streptomycin was added for 37°C incubation at 5% CO_2 with saturated humidity for passage. Cells were randomly assigned into three groups: control group, NC group that was transfected with lncRNA MALAT1 NC control sequence, and lncRNA MALAT1 siRNA group which was transfected with lncRNA MALAT1 siRNA into endometrial cells.

Liposome Transfection of lncRNA MALAT1 siRNA into Endometrial Cells

lncRNAMALAT1 siRNA (5'-AAUCG UUGUG UGGGC ACA-3') and negative control (NC) sequences (5'-AUAGU UGCCG UCGGA AUG-3') were transfected into endometrial cells. In brief, cells were cultured until 70-80% confluence. lncRNA MALAT1 siRNA and NC liposome were added into 200 µl serum-free DMEM medium for 15 min room temperature incubation after complete mixture. Lipo2000 mixture was mixed with mir-33b mimics or mir-33b inhibitor plus NC for 30 min room temperature incubation. Serum was removed from cell culture and cells were rinsed gently in PBS. 1.6 ml serum-free DMEM medium was added into all systems for 37°C incubation for 6 h in a 5% CO_2 incubator. Serum-containing DMEM medium was switched for 48 h continuous incubation.

Real-Time PCR for Measuring lncRNA MALAT1 Expression in Endometrial Tissues and Cultured Cells

Trizol reagent was used to extract mRNA from Ems and normal endometrial tissues, and *in situ* endometrial tissues. DNA reverse transcription was performed following the instruction of the test kit. Primers were designed based on Primer Premier 6.0 (Table I) based on target gene sequence. Real-time PCR was used for measuring target gene expression under the following conditions: 55°C for 1 min, followed by 35 cycles each consisting of 92°C 30 s, 60°C 30 s, and 72°C 30 s. Data were collected to calculate CT values of all standards and test samples based on fluorescent quantification using GAPDH as the reference. Using CT values of standards, a standard curve was plotted for semi-quantitative analysis using $2^{-\Delta Ct}$ approach.

MTT Assay for the Effect on Cell Proliferation

Endometrial cells at log-growth phase were inoculated into 96-well plate using DMEM medium containing 10% FBS at 5 X 10^3 density. After 24 h incubation, the supernatant was discarded. 20 µl sterile MTT was added into each well at 24 h time interval. Triplicated wells were set for each time point. After 4 h continuous incubation, the supernatant was completely removed, and 150 µl DMSO was added into each well for 10 min vortex. After complete resolving of violet crystals, absorbance (A) values were measured at 570 nm wavelength. Proliferation rate = (A values of experimental group / A values of control group X 100%).

Caspase 3 Activity Assay

Caspase 3 activity was measured in all groups of cells following the manual instruction of test kit. In brief, cells were digested by trypsin, and the lysate was centrifuged at 600 g under 4°C for 5 min. The supernatant was discarded and cell lysate was added for 15 min iced lysis, followed by 20000 g centrifugation at 4°C for 5 min. 2 mM Ac-DEVD-pNA was then added, and optical density (OD) values at 405 nm wavelength were measured to calculate Caspase 3 activity.

Transwell Chamber Assay

Following the manual instruction, serum-free medium was added. After 24 h, the bottom and upper phase of the membrane were pre-coated with 50 mg/L Matrigel dilution (1:5), followed by air-drying at 4°C. 500 µl DMEM medium containing 10% FBS and 100 µl cell suspensions in serum-free DMEM were added into the interior and exterior of the chamber, using triplicated wells for each group. The chamber was placed into a 24-well plate, and control group utilized transwell chamber without Matrigel. After 48 h incubation, transwell chamber was washed in PBS to remove cells on the membrane. Cells were then fixed by cold ethanol. After staining in crystal violet, cells at the lower phase of the microspore membrane were enumerated. All experiments were repeated for three times.

Western Blot for Measuring Protein Expression of NF-κB, iNOS and MMP-9

Total proteins were extracted from all groups of endometrial cells. In brief, cells were lysed on ice for 15-30 min using lysis buffer. Cells were ruptured using ultrasound (5 s, 4 times), and were

Table I. Primer sequences.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
GADPH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
lnc RNA MALAT1	CCACATCACGGCTGTTCTTGTA	GCATTGTGTCGGCTGGTAATT

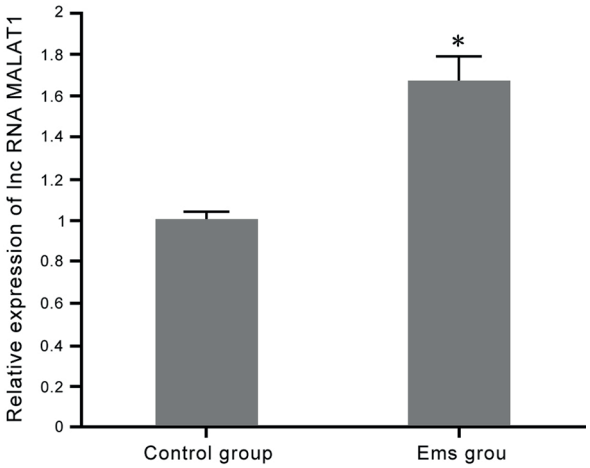

Figure 1. Differential expression of lnc RNA MALAT1 between Ems and *in situ* endometrial tissues. *p<0.05 *vs.* control group.

centrifuged at 10 000 g for 15 min at 4°C. The supernatant was saved and proteins were quantified using Bradford method for storage at -20°C in Western blot. Proteins were separated using 10% SDS-PAGE and were transferred to PVDF membrane using semi-dry method (200 mA, 2 h). Non-specific background was removed using 5% defat milk powder for 2 h room temperature incubation. Monoclonal antibody against NF-κB (1:1000), iNOS (1:2000), and MMP-9 (1:2000) was added for 4°C overnight incubation. On the next day, the membrane was washed in PBST followed by 30 min room temperature incubation in 1:2000 diluted goat anti-rabbit secondary antibody. After PBST rinsing, chromogenic substrate was added for 1 min development, followed by

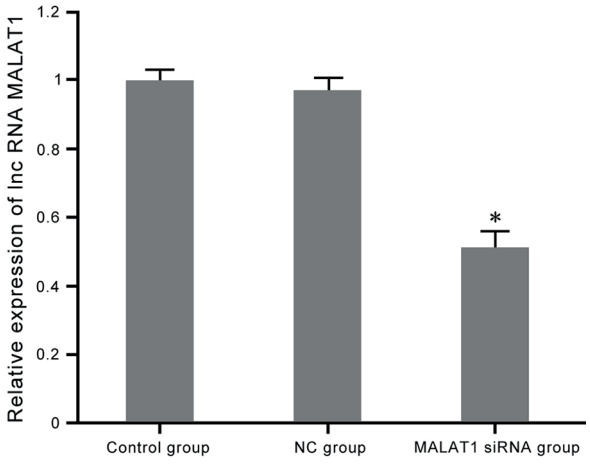

Figure 2. Regulation of lnc RNA MALAT1 in endometrial cells. *p <0.05 *vs.* control group.

X-ray exposure. Protein imaging processing software and Quantity one software was used to scan X-ray film and to measure the band density. All experiments were repeated for four times for the statistical analysis.

Statistical Analysis

All data were presented as mean± standard deviation (SD). Comparison of means between two groups was performed by *t*-test. SPSS 11.5 software was used for statistical analysis. Difference among groups was analyzed by one-way analysis of variance (ANOVA). A statistical significance was defined when p<0.05.

Results

Differential Expression of lncRNA MALAT1 in Ems and In Situ Endometrium

Real-time PCR was used to analyze differential expression of lncRNA MALAT1 between Ems and *in situ* endometrial tissues. *In situ* endometrium (control group) showed relatively lower lncRNA MALAT1 expression, whilst Ems group had significantly enhanced lncRNA MALAT1 expression (p<0.05, Figure 1).

Regulation of lncRNA MALAT1 Expression in Endometrial Cells

The transfection of lncRNA MALAT1 siRNA into endometrial cells significantly suppressed the expression of lncRNA MALAT1 (p<0.05 comparing to control group). The transfection of lncRNA MALAT1 NC into endometrial cells (NC group) did not affect lncRNA MALAT1 expression without statistical significance compared to the control group (Figure 2).

Effects of lncRNA MALAT1 on Endometrial Cell Proliferation

The transfection of lncRNA MALAT1 siRNA into endometrial cells to suppress its expression can significantly inhibit endometrial cell proliferation (p<0.05 comparing to control group, Figure 3).

Effects of lncRNA MALAT1 Regulation on Endometrial Cell Invasion

Transwell chamber assay was used to measure the effect of lncRNA MALAT1 on invasion of endometrial cells. The transfection of lncRNA MALAT1 siRNA into endometrial cells significantly inhibited endometrial cell invasion (p<0.05 comparing to control group, Figure 4).

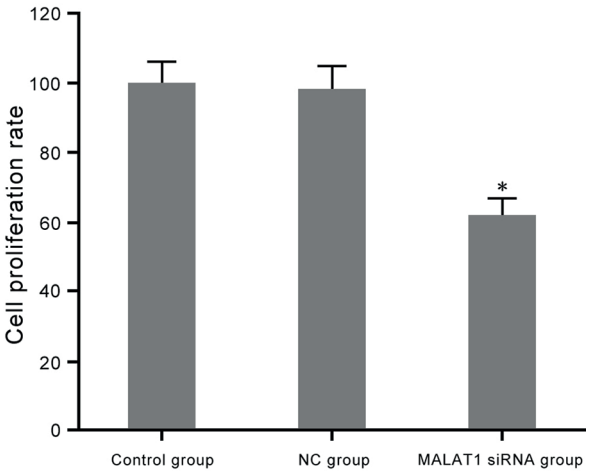

Figure 3. The effect of lnc RNA MALAT1 regulation on endometrial cell proliferation. *p<0.05 *vs.* control group.

Effects of lncRNA MALAT1 on Apoptosis of Endometrial Cells

Caspase3 activity assay was employed to analyze the effect of lncRNA MALAT1 regulation on apoptotic activity of endometrial cells. The transfection of lncRNA MALAT1 siRNA into endometrial cells suppressed its expression and facilitated Caspase3 activity in endometrial cells (p<0.05 comparing to control group, Figure 5).

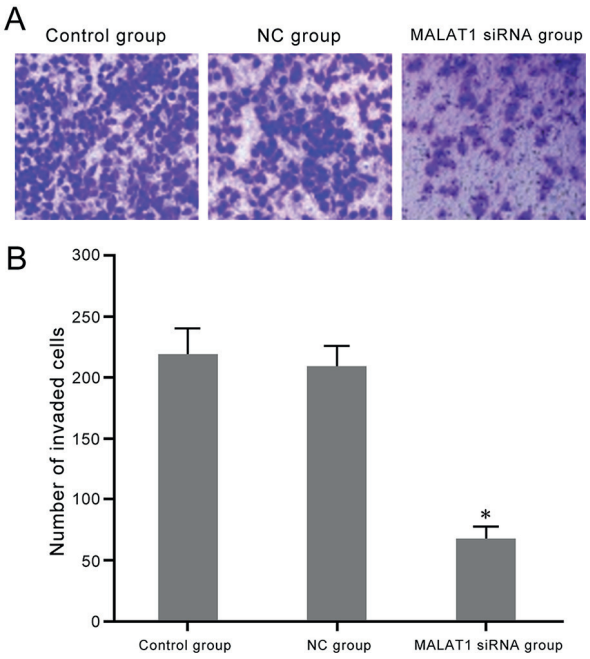

Figure 4. Effect of lnc RNA MALAT1 regulation on endometrial cell invasion. ***A*,** Transwell chamber assay for the effect of lnc RNA MALAT1 on endometrial cell invasion. ***B*,** Analysis for the effect of lnc RNA MALAT1 on the invasion of endometrial cells. *p<0.05 *vs.* control group.

Effects of lncRNA MALAT1 Regulation on NF-κB/iNOS Signal

Western blot was used to analyze the effect of lncRNA MALAT1 regulation on NF-κB/iNOS signal. The transfection of lncRNA MALAT1 siRNA into endometrial cells suppressed its expression and decreased NF-κB or iNOS protein expression (p<0.05, Figure 6).

Effects of lncRNA MALAT1 on MMP-9 in Endometrial Cells

The transfection of lncRNA MALAT1 into endometrial cells to suppress its expression significantly inhibited MMP-9 expression (p<0.05 comparing to control, Figure 7).

Discussion

Differential expression of lncRNA is under the regulation by various factors including transcriptional regulation, pathological status, and environmental adaptation. Therefore, lncRNA may present abnormal expression across tissues and cells, or even within the same tissues, with effects on both expressional and regulatory mechanisms[17]. As one of the most common disorder in gynecology, Ems has been found to be associated with differential expression of lncRNA[17,18]. lncRNA MALAT1 participates in cell metabolism, cell proliferation, and invasion modulation, and plays important roles in tumor and metabolic

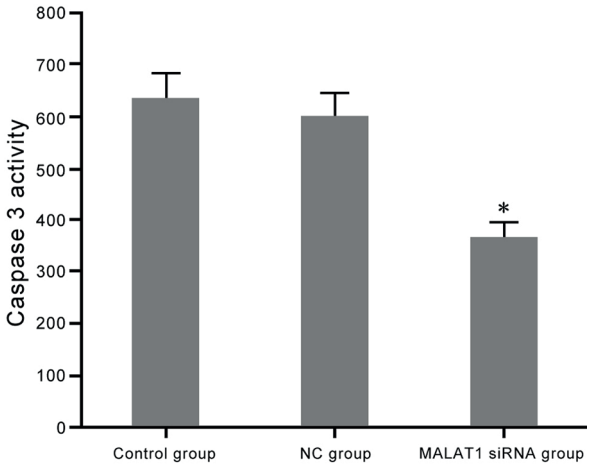

Figure 5. The effect of lnc RNA MALAT1 on apoptotic activity of endometrial cells. *p<0.05 *vs.* control group.

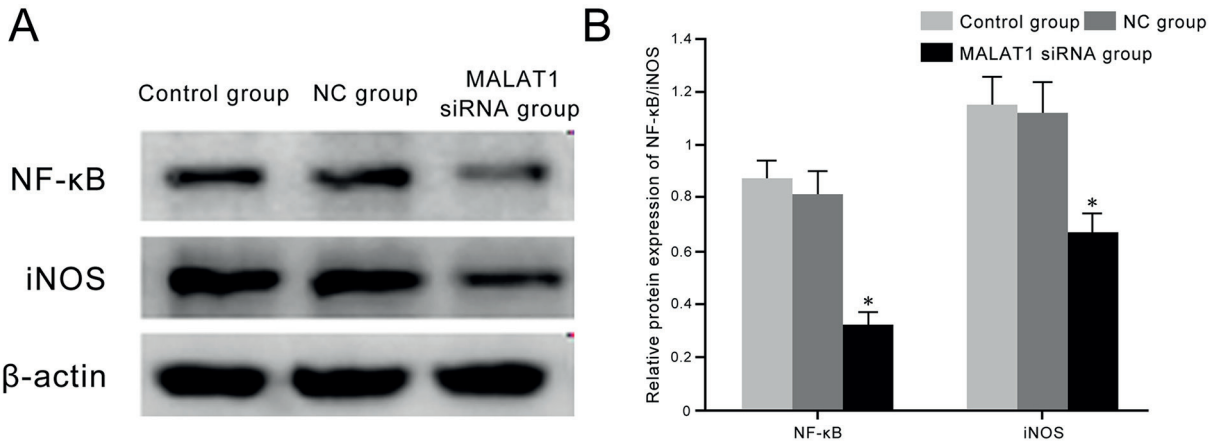

Figure 6. Regulation of lnc RNA MALAT1 on NF-**κB**/iNOS signal. ***A,*** Western blot bands for the effect of lnc RNA MALAT1 on NF-κB/iNOS signal of endometrial cells. ***B,*** Analysis for the effect of lnc RNA MALAT1 on NF-κB/iNOS signal. *$p<0.05$ *vs.* control group.

disorders[19]. Thus, this study firstly measured the expression of lncRNA MALT1 in Ems and found significantly higher lncRNA MALAT1 in Ems comparing to *in situ* endometrial tissues.

We further analyzed the effect of lncRNA MALAT1 expression regulation on endometrial cells and confirmed that lncRNA MALAT1 siRNA transfection into endometrial cells for suppressing its expression could inhibit *in situ* endometrial cell proliferation or invasion, plus enhanced Caspase 3 activity. As one of the most potent members in apoptotic family, Caspase 3 activity enhancement can induce cell apoptosis[20]. This study suggests that regulation of lncRNA MALAT1 expression could facilitate endometrial cell apoptosis, for further inhibition on endometrial cell proliferation or invasion. As one nuclear transcription factor, NF-κB participates in inflammatory response and immune reaction or other physiological processes. The co-activation of NF-κB and inducible nitric oxide synthase (iNOS) that are related to immunity or inflammation also exerted important roles[21]. Previous studies showed that enhanced expression of iNOS could facilitate angiogenesis, resist cell apoptosis and accelerate cell proliferation[22]. MMP-9 participates in various body pathological and physiological processes and plays important roles in cell proliferation and migration[23]. Moreover, it demonstrates that by interference of lncRNA MALAT1 expression, one can downregulate NF-κB/iNOS signal pathway for further inhibition on MMP-9 expression, thus participating in Ems progression regulation. However, the current study only investigated the

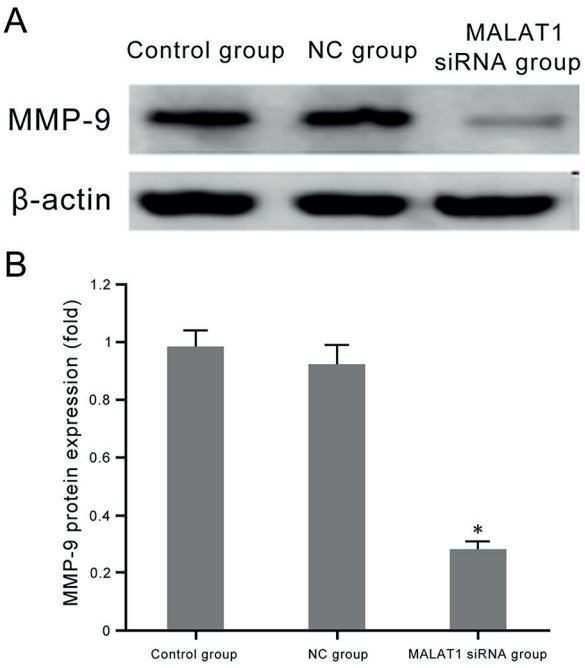

Figure 7. Effects of lnc RNA MALAT1 regulation on MMP-9 in endometrial cells. **A,** Western blot for the effect of lnc RNA MALAT1 on MMP-9 in endometrial cells. **B,** Analysis for the effect of lnc RNA MALAT1 on MMP-9 expression in endometrial cells. *$p<0.05$ *vs.* control group.

differential expression of lncRNA MALAT1 in clinical samples and analyzed his role in Ems by *in vitro* assay of lncRNA MALAT1. Further studies can be performed to investigate the functional target of lncRNA MALAT1 and to analyze detailed mechanism via *in vivo* assay, in order to

provide evidences for illustrating pathogenesis mechanism of Ems and treatment.

Conclusions

Our results showed that LncRNA MALAT1 can facilitate endometrial cell apoptosis via NF-κB/iNOS pathway and change MMP-9 expression for suppressing endometrial cell proliferation or invasion, modulating Ems pathogenesis.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by the Fujian Provincial Natural Science Foundation Youth Innovation Project (No. 2018J05126).

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