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Regulation of miR-33b on endometriosis and expression of related factors

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Abstract. – OBJECTIVE: Endometriosis is a common benign disease in gynecology, and can cause chronic pelvic pain, dysmenorrhea and even infertility. Its pathogenesis mechanism has not been fully illustrated. miRNA (miR) participates in various biological activities including cell growth, proliferation, apoptosis, organ formation, inflammation and tumor. Its role in endometriosis has not been reported. MiR-33b is involved in cell metabolism, proliferation and invasion, but with its function and mechanism in endometriosis unknown.

PATIENTS AND METHODS: Real-time PCR was used to test miR-33b expression in ectopic endometrial and normal tissues. *In vitro* cultured endometrial cells were tra ed with miR-33b mimic or inhibitor, fo ed by Real-time PCR for miR-33b expression. T method detected endometrial cell proliferat Caspase 3 activity was quantified by test kit. l-time PCR and Western blot ed effe of miR-33b on vascular end wth fac tor (VEGF) and matrix met rotein MMP-9).

RESULTS: MiR-33b wa wn-regu in ectopic endometrial tiss s 05 normal tissues). Tran tion 3b inhibitor facilitated endom prolife decreased Caspase 3 activ reased VE d MMP-9 mRNA or prot ion ($p < 0.$ mpared to control gro y. MiR imic suppressed endometrial iferation, e d Caspase 3 activity, and eased VEGF o -9 expression ($p < 0.05$ pared to control gr p).

C LUSIO MiR-33b can mediate cell apo s, VEGF and MMP-9 expression and a liferati nd apoptosis of uterus ome ells. participating endometri- orma

Ke rds: , MicroRNA-33b, Cell proliferation, GF, Mi

Introduction

Endometriosis is a common and frequent benign disease in gynecology[1]. With rapidly increasing incidence, lom osis s verely affects women h ily lif Some endometriosis ons may lom nor-like infiltrative behavior, out 1.0% probabili m ant transfo iation and a certain rate of rec ce, therefore drawing incr research i ts[3,4]. Endometriosis is nifested as implan ion of endometrial t es on ectopic side, such as extra-muscular l of uterus uding ovarian, rectum-uterus pression bladder-uterus depression[5]. End riosis ds to chronic pelvic pains, dysme menoxenia and even infertility[6]. Currently both medication and surgery are le for treating endometriosis. Drugs can app endometriosis to certain extents, but having defects such as adverse reactions and higher recurrence[7]. Radical surgery is usually applicable for fertile women, whilst conservative surgery also faces the issue of high recurrence[8]. EMS has a complicated pathogenesis mechanism, which has not been fully illustrated[9]. Therefore, the identification of endometriosis pathogenesis related molecular targets can benefit treatment efficacy of endometriosis[10]. MicroRNA (MiR) is widely distributed in animal/plant cells and even virus, with 22-23 nucleotides length having endogenous regulatory role[11]. MiR can negatively regulate gene expression via complementary binding with target mRNA for degradation of mRNA or post-transcriptional inhibition[12]. MiR participates in various biological activities, including cell growth, proliferation, apoptosis, organ formation, inflammation and tumor[13,14]. The role of miR in endometriosis has not been reported. MiR-33b plays a role in cell metabolism and regulation of cell proliferation or invasion, which is important for diseases, such as tumor or metabolic disorder[15,16]. However, its expression or functional role in endometriosis has not been fully illustrated.

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Patients and Methods

Recruitment of Research Objects and Sample Collection

Endometriosis patients who were admitted in Renmin Hospital of Wuhan University from January 2015 to June 2016 were recruited, in parallel with 15 patients having uterus prolapse, hysteromyoma or benign ovarian tumors as the control group. Patients aged between 37 and 47 years (average age: 38.6 ± 8.2 years). A total of 20 patients who were confirmed as endometriosis and having surgery in our hospital were recruited as endometriosis group (aging between 36 and 50 years, average age: 47.2 ± 9.8 years). No significant difference existed in general information between these two groups, so comparison was possible. All patients have not received related treatment before, nor did any chemo-, radio- or hormone therapy. No antibiotics have been applied within 6 months before surgery. No patients had intrauterine device implantation. Those patients with severe organ failure, malignant tumor or severe complications were excluded. Endometrial tissue collected during surgery were partially cult Dulbecco's Modified Eagle's Medium (DM (Hyclone, Logan, UT, USA) or frozen in -8 fridge for further use. The study protocol w approved by the Research E committe of Renmin Hospital of Wu Un ity and all patients gave their in ed con t before study commencement.

Major Reagents d Equip t

Dulbecco's ified Eag Medium (DMEM), fet ov rum (FBS), nd streptomycin-pe icillin we rchased from Hyclone (L , UT, USA). ethyl sulphoxide (DMS and 4,5-dimethyl- niazolyl)-2,5-diphe -H-te lium bromide (MTT) powders p ased f m Gibco (Grand Island, NY, U ypsin A lysis buffer was pur- d f Sig Aldrich (St. Louis, MO, U Capa ctivity assay kit and polyvinyl e fluorid (PVDF) membrane were pur- all Life Sciences Inc. (Pensacola, , USA). Ethylene diamine tetra acetic acid DTA) was purchased from Hyclone (Logan, USA). Western blotting reagent was purch d from Beyotime (Beijing, China). *Enhanced* chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Piscataway, NJ, USA). Rabbit anti-human vascular endothelial growth factor (VEGF) monoclonal antibody (Catalogue No. 2445; 1:3000), rabbit anti-human matrix metalloprotein 9 (MMP-9) monoclonal antibody (Catalogue No. 2270; 1:3000), and mouse anti-rabbit hor peroxidase (HRP)-conjugated IgG nda antibody (Catalogue No. 7074; 00) were all purchased from Cell Signa Technology (Beverly, MA, USA). TaqMan RNA reverse transcription kit purcha om Thermo Electron Corp tham, MA, MiR-33b mimic, miR- nhibit nd nega control (NC) sequen ver nthesi d by Gimma (Shangha hin extra kits and reverse t cription ver rchased from Axyg ksbury, M A). Other common ent purchase rom Sangon (Shanghai, China). stem Version 1.3.1 microp der was p ased from Bio-Rad L atories (Hercules, A, USA). ABI7700 fluorescent quantitative PCR cycler was p hased from BI (Foster City, CA, USA). U ure worl tion was purchased from Su h-te laterials Co. Ltd. (Shanghai, China). Scientific Forma CO_2 incubator was purchased from Thermo Electron Corp m, MA, USA). GeneAmp PCR system 400 was purchased from PE Applied Biosystems (Foster City, CA, USA).

Endometrial Cell Primary Culture and Grouping

Endometrial tissues were rinsed in sterile phosphate buffered saline (PBS) for 2-3 times, and were cut into 0.5-1.0 cm^3 tissue blocks, which were digested with 0.25% trypsin, 0.1% collagenase IV and 0.1% hyaluronidase at 37°C for 60 min. Lysate was centrifuged at 1000 r/min for 5 min centrifugation to discard the supernatant. Cells were re-suspended in 1 ml fresh DMEM, followed by 1000 r/min for 5 min centrifugation and discarding supernatant. The process was repeated and cells were re-suspended in 1 ml fresh DMEM and were cultured in 5 ml culture dish at 1×10^6 cells per cm^2, with addition of 2 ml fresh medium (90% high-glucose DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin and 10% fetal bovine serum (FBS) under 37°C culture with 5% CO_2 for 24-48 h. Cells at 3-8 generation at log-growth phase were selected and randomly divided into four groups: miR-33b mimic NC, miR-33b inhibitor NC, miR-33b mimic and inhibitor group.

Liposome Transfection of miR-33b Mimic and miR-33b Inhibitor

MiR-33b mimic (5'-AGGAU CGGUU UGUGC ACA-3'), miR-33b inhibitor (5'-AUCGG AUGUG GUGCA CUA-3'), miR-33b mimic NC (5'-AUUUG CCAGG UCGGA AUG-3') or miR-33b inhibitor NC (5'-AGGUC AAGCA GUUCG UUG-3') were transfected into endometrial cells. In brief, cells were cultured until reaching 70-80% confluence. MiR-33b mimic/inhibitor or negative control liposome were mixed with 200 µl serum-free medium for 15-min room temperature incubation. Lipo2000 reagent was then mixed with miR-33b mimic, miR-33b inhibitor or negative controlled dilutions for 30-min room temperature incubation. Serum was removed, followed by phosphate buffered saline (PBS) rinsing gently and the addition of 1.6 ml serum-free culture medium. Cells were then kept in a humidified chamber with 5% CO_2 at 37°C for 6 h, followed by the application of 10% fetal bovine serum (FBS)-containing medium in 48 h continuous incubation for further experiments.

Real-time PCR for Detecting miR-33b VEGF and MMP-9 Expression in Endometrial Tissues

Trizol reagent was used to extract RNA from normal and ectopic endometrial tissues. Reverse transcription was performed according to the manual instruction of test kit, using primers designed by PrimerPremier 6.0 (Table I). Real-time PCR was performed on target genes under the conditions: 55°C for 1 min, pre-denaturation by 35 cycles each containing 92°C for 30 s, 58°C for 45 s and 72°C for 35 s. Data were collected and calculated for CT values of all samples and standards based on fluorescent quantification using GAPDH as the reference. Standard curve was firstly plotted using CT values of standards, followed by semi-quantitative analysis by $2^{-\Delta\Delta Ct}$ method.

MTT Assay for Cell Proliferation

Exponentially growing cells at log-phase were seeded into 96-well plate which contained Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at 5×10^3 density. After 24 h incubation, the supernatant was removed. After 24 h incubation, 20 µl sterile 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added in each well in triplicates. After 4 h continuous culture, the supernatant was completely removed, with the addition of 150 µl dimethylsulfoxide (DMSO) for 10 min vortex until the complete dissolving of crystal violet. Absorbance (A) values were measured at 570 nm in micro-plate reader. The proliferation rate was calculated for each group.

Caspase 3 Activity Assay

Caspase 3 activity in cells was evaluated using test kit from three groups. In brief, cells were digested with trypsin and were centrifuged at 600 ×g for 5 min under 4°C. The supernatant was discarded, followed by the addition of cell lysis buffer and ice-bath incubation for 15 min. The mixture was then centrifuged at 20 000 ×g for 5 min under 4°C, followed by the addition of 2 mM Ac-DEVD-pNA. Optical density (OD) values at 450 nm wavelength were measured to reflect Caspase 3 activity.

Western Blot for VEGF and MMP-9 Protein Expressions

Total proteins were extracted from endometrial cells. In brief, cells were lysed on ice for 15-30 min, with ultrasound treatment (5 s, 4 times). After centrifugation at 10 000 ×g for 15 min, the supernatant was saved, quantified by Bradford method and was stored at -20°C for Western blot assay. Proteins were separated in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and were transferred to polyvinylidene fluoride (PVDF) membrane by semi-dry method (100 mA, 1.5 h). Non-specific binding sites were removed by 5% defatted milk powder for 2 h. Anti-VEGF monoclonal antibody (1:1000), anti-MMP-9 monoclonal antibody (1:2000) were added for 4°C overnight incubation. After phosphate-buffered saline-tween (PBST) washing, goat

Table I. Comparison of POCD in two groups.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
miR-33b	ATTCTTTCGAACTGTCTTGG	TCACCCTCGGCTGTC CTGACA
VEGF	TGACACGGCTGTTCTTTC	CCGACTTTCGAGTCTT
MMP-9	CCACATCCGACTAGCTGTA	GCATTGTGTACCGCGGTAATT

anti-rabbit secondary antibody (1:2000) was added for 30 min incubation at room temperature. Electrochemiluminescence (ECL) reagent was then added for developing the membrane for 1 min after PBST rinsing, followed by X-ray exposure. The film was scanned and analyzed by protein imaging system and Quantity One software for measuring band density. Each experiment was replicated for four times (n=4) for statistical analysis.

Statistical Analysis

All data were presented as mean ± standard deviation (SD). Comparison of means between groups was performed by Student's *t*-test. SPSS 11.5 software (SPSS Inc., Chicago, IL, USA) was used for analyzing data. Comparison between the groups was made by analyzing the data with Turkey's post hoc test. Statistical significance was defined when $p < 0.05$.

Results

Expressional Profile of miR-33b in Ectopic and Normal Endometrial Tissues

Real-time PCR was used to analyze ... pressional difference between ectopic and n... endometrial tissues. Results showed relati... higher miR-33b in control group whilst endo... triosis tissues had lower miR-33... ssion (p < 0.05, Figure 1).

Effects of miR-33b on ... Endometrial Tissu...

RT-PCR was us... test the ... of miR-33b mimic or inhib... nsfection ... expression in endo... al ... s. The tra... ection of

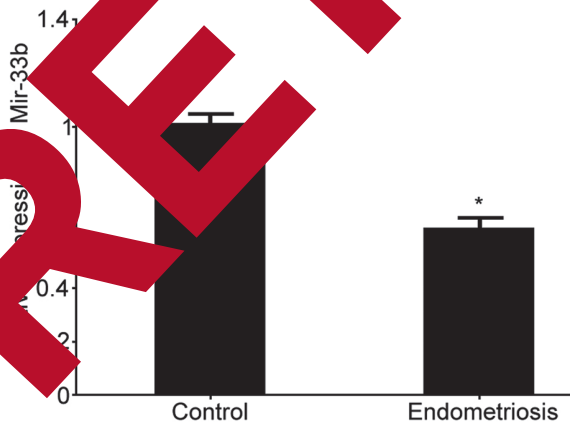

Figure 1. Expression of miR-33b in endometriosis and normal tissues. *$p < 0.05$ compared to control group.

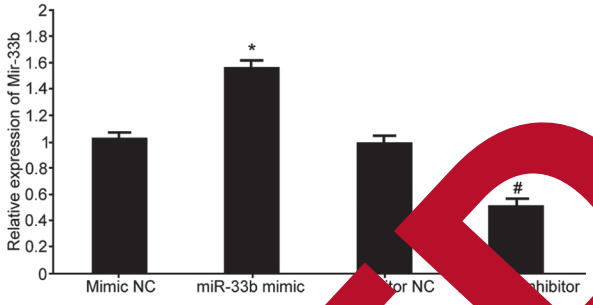

Figure 2. MiR-33b expression ... ndometrial tiss... transfection. *$p < 0.05$ comp... o mimi... C group; ... 0.05 compared to inhibito... roup.

miR-33b mimi... nificantly ...ilita... miR-33b expression (... 5 compared ...rol group), whilst mi... b ...itor transfe...on inhibited miR-33b expression ... 0.05 compared to control ... igure 2).

...cts of miR-33b on Endometrial Cell ...liferation

...T assay w... sed to test the effect of miR-33b ...ic/inh... r transfection on proliferation of end... tissues. After transfection using miR-33b mimic to facilitate its expression, proliferation of endometrial cells was suppressed (p < 0.05 ...pared to control group). The transfection of miR-33b inhibitor to down-regulate its expression could facilitate endometrial proliferation (p < 0.05 compared to control group, Figure 3).

MiR-33b regulation and Caspase 3 Activity in Endometrial Tissues

The transfection of miR-33b mimic facilitated Caspase 3 activity ($p < 0.05$ compared to control group). The transfection of miR-33b inhibitor to down-regulate its expression suppressed Caspase 3 activity ($p < 0.05$ compared to control group, Figure 4).

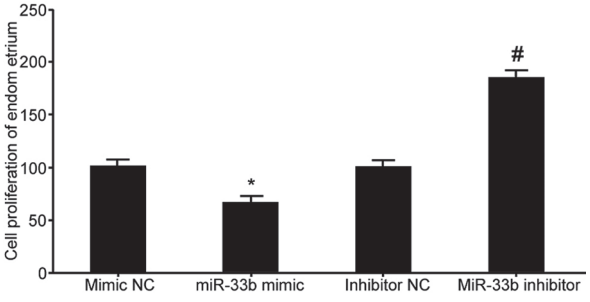

Figure 3. Effects of miR-33b on proliferation of endometrial cells. *$p < 0.05$ compared to mimic NC group; #, $p < 0.05$ compared to inhibitor NC group.

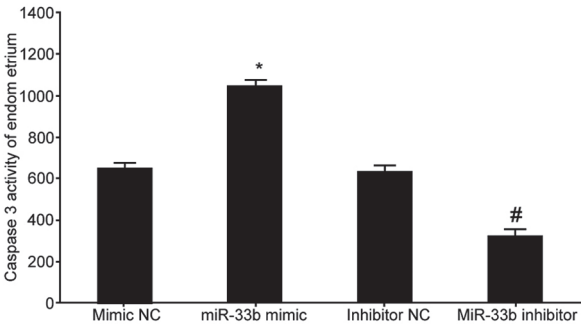

Figure 4. Effects of miR-33b mediation on Caspase 3 activity in endometrial cells. *$p < 0.05$ compared to mimic NC group; #, $p < 0.05$ compared to inhibitor NC group.

Effects of miR-33b on VEGF Expression in Endometrial Cells

Real-time PCR and Western blot were used to test the effect of miR-33b on vascular endothelial growth factor (VEGF) mRNA and protein expression. The transfection of miR-33b mimic decreased vascular endothelial growth factor (VEGF) mRNA and protein expression ($p < 0.05$ compared to control group). The transfection of miR-33b inhibitor facilitated VEGF mRNA/protein expression ($p < 0.05$ compared to group, Figure 5).

Regulation of miR-33b and MMP-9 Expression in Endometrial

Real-time PCR and West blo re used to quantify the effect of -33b MMP-9 mRNA/protein expressio endo Transfection of miR- mi used M -9 mRNA and prot expressio < 0.05 compared to control Transfecti miR-33b inhibitor faci d -9 mRNA d protein expression < 0.05 c ed to control group, Figure 6

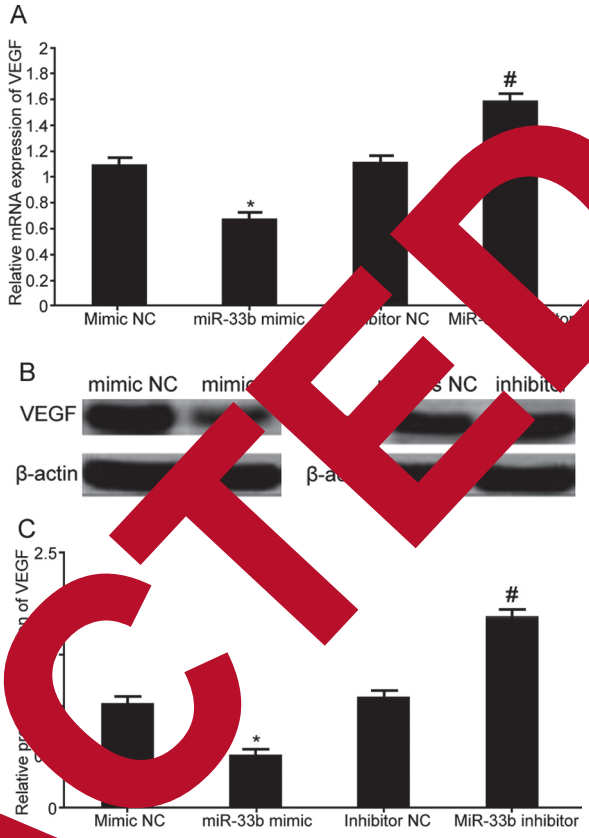

Figure 5. Effects of miR-33b modulation on VEGF expression in endometrial cells. ***(A)*** Real-time PCR for VEGF mRNA expression; ***(B)*** Western blot for VEGF protein expression in endometrial tissues; ***(C)*** Analysis of miR-33b effects on VEGF expression. *$p < 0.05$ compared to mimic NC group; #, $p < 0.05$ compared to inhibitor NC group.

Discussion

icro ha ghly conserved structure, tis specifi ain, temporal expressional pr and va able structure[15]. MicroRNA as nucleotide has widely regulatory nctions, and plays a critical role in cell prolif- tion, differentiation, apoptosis and immune nse[16]. Expressional profile of microRNA is un r the regulation of multiple factors including transcriptional regulatory level, physiological status and environmental change. Therefore, in different tissue/cell types, microRNA expression and regulation mechanism are under influence[17]. MicroRNA is closely correlated with disease type, and can be used as the important target for disease diagnosis and prognosis, thus becoming a major challenge for modern medicine[18]. MiR-33b as one newly discovered microRNA, has been shown to have various physiological/pathological activities, and is involved in metabolic diseases in addition to tumor oncogenesis[19,20]. In endometriosis, which is the most common disorder in gynecology, microRNA expressional profile has been shown to have difference, but leaving the role of miR-33b no fully illustrated[21]. Therefore this study investigated the expressional profile of miR-33b in endometriosis, and found lower miR-33b expression in endometriosis tissues than controlled endometrial tissues. By mediating miR-33b expression, we confirmed that transfection of miR-33b inhibitor inhibited its expression,

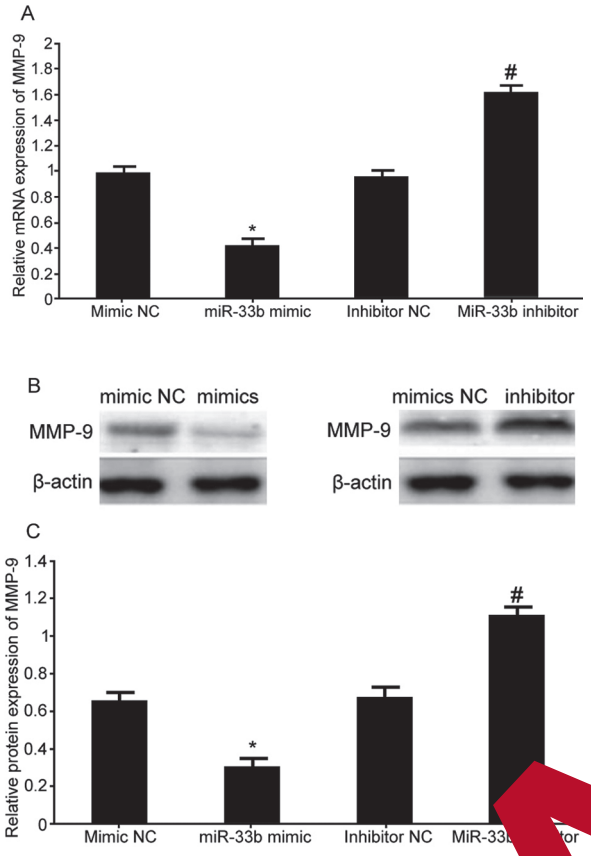

Figure 6. MiR-33b regulation and MMP-9 expressio endometrial cells. ***(A)*** Real-time PCR f MP-9 mR expression; ***(B)*** Western blot for MM expressio in endometrial tissues; ***(C)*** Ana of m b effects on MMP-9 expression. *$p < 0$ mpared imic NC group; #, $p < 0.05$ compared to tor NC

facilitated the p tion of end rial cells, and decrease asp activity. nsfection of miR-33b mimic faci d its expression, inhibited e metrial cell p ation and elevated Ca e 3 activity. Thes esults suggested that -33b d regulate endometrial tissues pro ion medi g apoptosis. As one of the mo ent ang esis facilitating factor, lar he growth factor (VEGF) is ex sed in ar endothelial cells, thus enha g vessel permeability, in addition to deg- a-cellular matrix, thus facilitating l proliferation, differentiation and migration[22]. trix metalloproteinase (MMPs) participate in gulation of various body pathophysiological pr esses, and play positive roles in embryonic implantation or wound healing. It is also involved in other diseases such as inflammatory response, autoimmune disease, invasion or metastasis of malignant tumors, and cardiovascular disease[23,24]. MMP-9 has been shown to play an important role in cell proliferation and migration[25,26]. Further study found that transfection of miR-33b inhibitor elevated VEGF and MMP-9 mRNA o expression, whilst miR-33b mimic t ect suppressed mRNA/protein expres of VEGF and MMP-9.

Conclu ns

MiR-33b can affect fera and ap ptosis of endometrial ce ia g apo s and altering VEGF MMP-9 ssi us can work as a no t for diagn treatment of endome sis.

C ct of Interest

T uthors declare that they have no conflict of interests.

eferences

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