miR-218-5p in Endometrial Microenvironment Prevents the Migration of Ectopic Endometrial Stromal Cells by Inhibiting LASP1

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Abstract

Our previous two-dimensional electrophoresis experiments showed that the expression of LASP1 in patients with endometriosis was significantly higher than that of control endometrium. However, the molecular mechanism by which LASP1 is regulated in endometriosis/adenomyosis is unknown. Herein, qPCR was performed to analysis the expression levels of LASP1 and miR-218-5p between EMs cells and control cells. Immunofluorescence in situ hybridization was carried out to measure the expression level of miR-218-5p in ectopic endometrium versus normal endometrium. After miR-218-5p mimic or inhibitor were transfected, the transwell experiment was carried out to see the effect of miR-218-5p on the migration of ESCs. EdU was used to measure cell proliferation rate. Dual-luciferase reporter assay (DR) was used to verify the binding of hsa-miR-218-5p to the 3'UTR of LASP1. Western blot and immunofluorescence analysis were carried out to identify the protein expression pattern of LASP1 and EMT markers in endometrial tissue. Our results show that high expression of LASP1 is related to the development of EMs. miR-218-5p inhibits the expression level of LASP1 by binding the 3'UTR region of LASP1. miR-218-5p is negatively correlated with the expression of LASP1 protein in endometriosis cells and tissues. Without affecting proliferation, miR-218-5p can increase the migration of ESCs. miR-218-5p is mainly secreted from blood vessels and expressed in the muscle layer around the endometrium. miR-218 also inhibits mesenchymal transition of epithelial cells by inhibiting the expression of Vimentin.

Introduction

Although endometriosis/adenomyosis (EMs/ADs) are benign diseases, most studies have found that the infiltration and metastasis characteristics of EMs are similar to malignant tumors. Studies have found that the ability of metastatic bladder cancer cell lines to infiltrate and metastasize is similar to that of endometrial cells entering the myometrium(Gaetje, Kotzian, Herrmann, Baumann, & Starzinski-Powitz, 1995). The key point of the infiltration and metastasis of cells is the migration and movement of cells, and the first step of migration is the remodeling of the filamentous actin cytoskeleton, which can promote the formation and adhesion of synapses(Yamaguchi & Oikawa, 2010).

Actin backbone protein LIM and SH3 protein1 (LASP1) is a special actin binding and plaque binding scaffold protein, which participates in the migration of cells and plays a critical role in actin assembly, such as adhesion spots, pseudopods and fragmented feet concentrated. The LIM domain at the amino terminus of the LASP1 protein is a zinc finger domain, which is mainly found in transcription factors, signal transduction proteins, and cytoskeleton proteins. As a regulatory binding domain, the LIM domain can mediate between protein-protein interaction. There are two nebulin-like repeats after the zinc finger structure region of LASP1 protein, named R1 and R2 region respectively. As the actin binding region of LASP1, R2 mediates the interaction between LASP1 and the actin at the protrusions of the cell membrane (Mihlan et al., 2013; Raman, Sai, Neel, Chew, & Richmond, 2010; Stolting et al., 2012; Wang, Feng, Xiao, & Ren, 2009), thereby stimulating the movement of cells and promoting cell migration. LASP1 was first found in breast cancer, and was confirmed to be related to the progression and metastasis of breast cancer(Wang, Zheng, Shen, & Shi, 2012). Other studies have confirmed that LASP1 is highly expressed in

a variety of malignant tumors such as ovarian cancer, liver cancer, colorectal cancer, and pancreatic cancer, and is closely related to the occurrence, development, invasion and metastasis of tumors, and is regarded as an oncogene(Latronico & Condorelli, 2015; Wang, Li, Jin, Cui, & Zhao, 2013b; Wang et al., 2014; Zhao et al., 2015).

MicroRNA (miRNA), which is about 20-25nt long non-coding RNA(Gottlieb & Pourpirali, 2016), is a single-stranded non-coding small RNA and has post-transcriptional regulatory activity(Benaich et al., 2014). Studies have found that LASP1, as a miRNA target gene, is involved in the pathophysiological process of multiple tumors(Hailer et al., 2014). For example, miR-203 acts on LASP1 to affect the proliferation, invasion, and metastasis of esophageal squamous cell carcinoma, prostate cancer, and human triple-negative breast cancer cells(Takeshita et al., 2012; Viticchie et al., 2011; Wan et al., 2014; Wang et al., 2013a). miR-1, miR-133a, miR-218-5p target down-regulation of LASP1 to inhibit the survival rate of bladder cancer cells(Chiyomaru et al., 2012).

Our primary data suggested that LASP1 in ectopic endometrial tissue of EMs patients was significantly up-regulated, which strongly suggests that LASP1 protein plays an important role in the development of EMs(Liu et al., 2021). At present, there is no relevant study on whether miR-218-5p plays a role in EMs, and whether LASP1 is related to the development of EMs. Our work can verify the regulatory relationship of miR-218-5p to the downstream target protein LASP1 in the pathogenesis of EMs, which can give us a deeper understanding of the role and molecular mechanism of miRNA in the development of EMs, providing new ideas for in-depth study of the mechanism of EMs and basic experimental basis for future diagnosis and treatment of EMs in the future.

Materials And Methods

1. Human endometrial stromal cells (ESCs)

All experimental tissue specimens were obtained from patients treated in Jiangxi Maternity and Child Health Hospital (January 2018-January 2019). The control ESCs/ ThESC (control group 1) was obtained from ATCC cell bank. Patients who underwent laparoscopic surgery for benign diseases such as simple cysts, the specimens were pathologically diagnosed as normal endometrial tissue in 5 cases, and then ESCs were extracted (named Zx). The average age of the above patients was 30–40 years old, with an average age of (37.55 ± 3.43) years. Z9 was successfully extracted and stably cultured as the control group 2. For patients with clinically confirmed EMs, specimens were collected during laparoscopic surgery. The specimens were pathologically diagnosed as EMs. Seven cases of occidental endometrium were collected, and then ESCs were extracted as the eutopic endometrium group (the cells were named Q-Zx). The age of the patients ranged from 25 to 40 years, with an average of (33.37 ± 3.28) years old. Successfully extracted and stably cultivated Q-Z1 as the EMs group. Similarly, extracted and stably cultured Q-Y19 as the ectopic group of EMs. For patients with clinically confirmed AMs, specimens were also collected during laparoscopic surgery. The specimens were pathologically diagnosed as AMs, and 3 cases of ectopic lesions were intima, and then ESCs were extracted (cell named X-Yx). The age of the

patients was 27–40 years old, with an average of (35.27 ± 3.74) years old. Successfully extracted and stably cultured X-Y19 as AMs ectopic group.

Endometrial tissue from patients without hormone treatment for > 3 months undergoing hysterectomy from the Jiangxi Maternal and Child Health Hospital (Nanchang, Jiangxi Province, China). The samples have been shown to be in the proliferative phase of the menstrual cycle in pathology and histology. The patients signed a written informed consent form prior to recruitment. This study is in line with the Helsinki Declaration and approved by the Ethics Review Body Committee of the Jiangxi Maternal and Child Health Hospital. Separation, characterization and culture of EESCs were performed as described previously(Li et al., 2019).

2. Isolation, culture and identification of ESCs

The tissues were cut into pieces and cultured in 10% FBS + DMEM/F12 containing 1 mg/ml type IV collagenase for 1.5 h at 37°C. After the tissue was digested and filtrated with a cell strainer, the tissue was centrifuged at 1000 rpm for 5 min. The pellet was resuspended and cultured with 10% FBS + DMEM/F12 supplemented with 100 IU/ml penicillin, 50 mg/ml streptomycin. In Supplementary data, cell characterization was assessed by immunofluorescence using anti-Vimentin (1:1000 dilution, Proteintech Group) and anti-E-cadherin (1:1000 dilution, BD Transduction Laboratories) antibodies.

3. Plasmids and miRNA

The mimic and inhibitor of miR-218-5p were synthesized by Ribo Biotechnology (Guangzhou, China). The of dual-luciferase reporter plasmid of LASP1 3'UTR widetype or mutant sequence (207bp) was subcloned into GV272 vector with the XbaI/XbaI restriction endonuclease by Genechem (Shanghai, China).

4. Western blot

Western blotting was performed as previously described (Zhang & et al., 2017). To measure the endogenous protein level, ESCs were transfected with control mimic/inhibitor or miR-218-5p mimic/inhibitor, respectively. After 48 hours, RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM 475 M NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1xRoche complete protease inhibitor mixture) was used to lysis cell. Protein (about 20–30 μg/line) was separated by SDS-PAGE gel and transferred onto a PVDF membrane (Millipore, CA, USA), then blocked with 5% milk powder, and incubated with anti-LASP1 antibody (1:1000 dilution, rabbit, ab191022, Abcam) and anti-Vimentin (1:2000 dilution, Cat No: 10366-1-AP, Proteintech) and overnight at 4°C. The membrane was washed three times with 1xTBS-T and incubated with horseradish peroxidase (1:5000) secondary antibody for 1 hour at room temperature. Protein bands were visualized by ECL system (Thermo Fisher Scientific, IL, USA). The protein expression levels were normalized to the expression of GAPDH(1:2000 dilution, Cat No:sc-47724, Santa Cruz Biotechnology) in each line.

5. Cell migration assay

Cell migration assay was carried out as described previously (Zhang et al., 2017). For assessing ESCs cell migration, $1x10^4$ cells in serum free media were seeded into the transwell inserts (Corning) and allowed to migrate toward 10% FBS-containing medium. Later, the cells in the transwell inserts were removed, and the inserts were washed in PBS three times. The migrated cells on the bottom of the insert were fixed with methanol solution followed by crystal violet (1%) staining. After washing the inserts three times with PBS, the inserts were allowed to air dry and pictures were taken using Olympus X71. Six independent fields were counted for each transwell and the average number of penetrating cells was represented in the graphs. For assessing cell invasion, $1x10^4$ cells in serum-free medium were seeded in the matrigel-coated transwell inserts (BD Bioscience). The cells were later processed similar to that of cell migration assay.

6. EdU incorporation assay

After with miRNA mimic or inhibitor transfection, ESCs cells were seeded onto 24 well plate. Next day, cells were incubated with 10 μM Edu for 6 hours, washed 3 times wtih 1xPBS, fixed in 3.7% formaldehyde for 15 min at room temperature, and followed by Edu insertion detection according to the manufacturer instructions(Guangzhou RiboBio Co., Ltd, China)(Jin et al., 2014).

7. Immunofluorescence

The paraffin-embedded adenomyosis tissue sections were baked in the oven at 65°C for 12h. After deparaffinization and blocking, the antigen–antibody reaction was incubated overnight at 4°C. Immunofluorescence staining with following antibodies: anti-Vimentin (Cat No: 10366-1-AP, Proteintech), anti-E-cadherin (Cat No: 610181, BD, Biosciences), anti-VEGF (Cat No: 19003-1-AP, Proteintech), anti-SMA(Cat No: 55135-1-AP, Proteintech). Fluorescence was visualized with Olympus X71.

8. RNA isolation and qPCR

We carried out the RNA isolation as described previously(Yue et al., 2014). The total RNAs were extracted from ESCs by using the RNAiso reagent (TaKaRa, Shiga, Japan). The reverse transcription reaction was also performed by using the PrimeScript reagent Kit (TaKaRa). Real-time quantification PCR reaction was carried out by using the SYBR Premix Ex TaqII (TaKaRa) on the 7500 Real-Time PCR System (Applied Biosystems) with primers for: RT-LASP1-F': AACGCACACTACCCCAAG, RT-LASP1-R': CCACTACGCTGAAACCTTTG. RT-Actin-F': ACCTTCTACAATGAGCTGCG, RT-Actin-R': CCTGGATAGC-AACGTACATGG. The miR-218-5p (MQPS0000843) and U6 (MQPS0000002) primers were purchased from Ribo Biotechnology (Guangzhou, China). Experiments were repeated at least three times.

9. Statistical analysis

Statistical analyses were carried out by using SPSS 24.0 software. Data were presented as mean ± SEM. To determine the statistical difference of target genes mRNA expression between control group and EMs group by using Student's t-test. All p-values were two-tailed, and p-values < 0.05 were considered to statistical significance(*).

Results

1.miR-218-5p inhibits LASP1 in ESCs

First, we used qPCR to detect the expression of miR-218-5p and LASP1 in different ESCs. We found that the expression level of miR-218-5p was the highest in ThESC(ThE) cells, followed by the eutopic stromal cells Q-Z1 of patients with cystic cysts, and the lowest expression in the two ectopic stromal cells Q-Y19 and X-Y19. On the contrary, in Q-Y19 and X-Y19 cells, the expression level of LASP1 was the highest, followed by Q-Z1, and the expression level of miR-218-5p in ThE was the lowest (Figure.1A and B). There is no difference in the expression of miR218 and LASp1 between Q-Y19 and X-Y19 cells. The above results indicate that miR-218-5p is lowly expressed in the eutopic mesenchymal cells of patients with non-endometriosis, is moderately expressed in the eutopic mesenchymal cells of patients with EMs, and is expressed at a high level in the ectopic mesenchymal cells of patients with EMs. Moreover, the expression pattern of LASP1 is opposite to miR-218-5p, showing a negative correlation.

Next, we transfected the mimics or inhibitors of miR-218-5p to detect its effect on the expression pattern of LASP1 in the cells with low or high concentrations of miR-218-5p, respectively. Only 50nM (not 20nM) miR-218-5p inhibitor decreased the level of miR-218-5p while increased the level of LASP1 in ThESCs (ThE) cell (Figure.1C and 1D). 50nM miR-218-5p mimic increased the level of miR-218-5p while decreased the level of LASP1 in both Q-Z1 and Q-Y19 cell (Figure.1E-H).

Similarly, we used western blot to detect the protein level of LASP1 in different ESCs cells. The result of protein expression level is: X-Y19 > Q-Z1 ≈ Q-Y19 > ThESC (Figure.2A), which echoes the results of qPCR (Fig. 1B). At the protein level, when miR-218-5p mimic was transfected into Q-Z1 cells (a cell with low expression of miR-218-5p), we found that the expression of LASP1 was decreased (Figure.2B). On the contrary, when miR-218-5p inhibitor was transfected into ThE cells (a cell with high expression of miR-218-5p), we found that the expression of LASP1 was increased (Figure.2C). The above results indicate that miR-218-5p indeed inhibit the expression of LASP1 in ESCs.

MicroRNA can inhibit the post-transcriptional activity of the target gene by binding to its 3'UTR. Therefore, we constructed the luciferase reporter gene plasmids of the wild type 3'UTR and the mutant 3'UTR of LASP1 (Figure.2D). Then, miR-218-5p was co-transfected with the above-mentioned reporter-gene plasmid into 293T cells for luciferase reporter gene activity assay. The results showed that miR-218-5p can inhibit the activity of the wt plasmid, but fail to change the activity of the mutant plasmid (Figure.2D) .

2. miR-218-5p has no effect on the proliferation of ESCs but can inhibit the migration of ESCs

In order to further study the function of miR-218-5p in ESCs, we transfected miR-218-5p inhibitor into ThE cells with high expression of miR-218-5p, and performed transwell assay and Edu insertion experiments to detect the changes of cell migration and proliferation respectively. The results showed that after the inhibitor of miR-218-5p was transfected, the migration of ThE cells increased (Figure.2E), but the proliferation of ThE cell did not altered (Figure.2G). On the contrary, we transfected the mimic of miR218 in Q-Z1 cells with low miR-218-5p expression, and also carried out transwell assay and Edu insertion

experiment to detect the changes of cell migration and proliferation. The results showed that after the mimic of miR-218-5p was transfected (Figure.2F), the migration of Q-Z1 cells increased, but proliferation of Q-Z1 cell remained unchanged (Figure.2H).

3. miR-218-5p is mainly expressed in the vascular endothelial cells and muscle layer near the endometrium

The above experiment show that miR-218-5p can prevent cell migration by inhibiting LASP1 in ESCs. So what is its expression and localization pattern in the endometrial tissue, and is there any difference in expression between the control endometrium and the endometrium of patients with EMs? Next, we performed RNA fluorescence in situ hybridization to detect the expression and localization of miR-218-5p in endometrial tissue. In the control eutopic endometrium tissue(CEu), which is very interesting that miR-218-5p is not expressed in endometrial layer (EmL), but mainly expressed in the muscle layer (MCL) close to the endometrium, Show a form of scattered distribution in the cytoplasm (Figure.3A). However, in adenomyosis eutopic (AEu) and ectopic(AEc) endometrial tissues, miR-218-5p showed low expression in both the endometrium layer and muscular layer (Figure.3B and 3C). These results indicate that miR-218-5p is upregulation in the MCL of CEu (Figure.3D). And we also found that miR-218-5p has strong signal around blood vessels (Figure.3E). We used VEGF-α and SMA antibodies to label smooth muscle (including vascular smooth muscle) and vascular endothelial, respectively. We found that miR-218-5p mainly co-localized with endothelial cells (Figure.3F), but not with smooth muscle cells (Figure.3F). These results suggest that miR-218-5p may be secreted by vascular endothelial cells.

4. Inhibition of miR-218-5p can hinder the epithelial-mesenchymal transition

LASP1 can increase tumor cell migration and invasion by promoting epithelial-mesenchymal transition(Wang et al., 2014; Zhong et al., 2019). We observed the expression and localization of LASP1 and Vimentin (a mesenchymal cell marker) in endometrial tissue. The results showed that the expressions of LASP1 and Vimentin in the eutopic and ectopic endometrial gland epithelial cells of patients with EMs were significantly elevated than those of the control endometrium (Figure.4A-4C). But there is no obvious difference between the three groups in the overall stromal cells, but the scattered and aggregated high expression in the AEc stromal cells (Figure.4D and 4E). The above results indicate that the endometrial epithelial cells of EMs already have mesenchymal characteristic. Since miR-218-5p can inhibit LASP1 (Figure.1 and 2), it should also have an effect on EMT. Therefore, we transfected miR-218-5p mimic into ThE cells to detected the expression of Vimentin by Western blot and found that high concentration of miR-218-5p (100nM) can significantly inhibit the protein expression level of Vimentin (Figure.4F).

5. Vimentin was high expression levels in adenomyosis epithelial cells

In order to investigate the increase of Vimentin in endometrial gland epithelial cells of EMs, we used Vimentin and E-cadherin (epithelial cell marker) antibodies to perform the immunofluorescence in ectopic and eutopic endometrial tissue sections of Adenomyosis patients. The Fig. 5A and 5B (Ectopic

Endometrial) showed that the expression level of Vimentin in stomal cells (R2) was significantly lower than that in epithelium (R3) and muscle layer (R1). Similarly, in eutopic endometrial tissue sections, the expression level of Vimentin in stomal cells (R2') was also lower than that in epithelium (R3') (Figure.5C and 5D). Simultaneously, the E-cadherin was only expressed in gland epithelial cells (Figure.5A and 5C). The above results indicate that both Vimentin and E-cadherin are highly expressed in the glandular epithelium. In addition, we also found that the expression level of Vimentin in stromal cells of the normal adenomyosis tissue is indistinguishable from that of the epithelial cells (Figure.5E and 5F). Taken together, when miR-218-5p was up-regulated, Vimentin in stromal cell were decreased, which was consistent with the expression pattern of Vimentin in tissue sections of CEu. Our results imply that miR-218-5p inhibitor resulted in adenomyosis epithelial cells transformed into stromal cells through a EMT process.

Discussion

As a member of the LIM protein subfamily, LASP1 protein, which interacts with the protruding actin on the cell membrane through the mediation of the LIM structure, can also play a role in the formation, extension and invasion of cell pseudopods through the SH3 region (Tomasetto et al., 1995). Relevant studies have confirmed that LASP1 is highly expressed in a variety of malignant tumors, and affects the development, invasion and metastasis of tumors. Although EMs are benign diseases, they have the characteristics of malignant tumor invasion and metastasis. Based on our preliminary data, we found that the expression level of LASP1 protein was significantly increased in ectopic ESCs of EMs patients. Endometrial tissue mainly contains epithelial cells and stromal cells (ESCs). The occurrence of EMS is largely dependent on ESCs. Therefore, it was speculated that LASP1 affects the migration of ESCs and promotes the development of EMs. Because AMs and EMs are both endometriotic diseases and are related, in order to fully illustrate the role of LASP1 on ESCs, this study also compared adenomyosis (AMs) ESCs with ovarian endometriosis(OEMs) ESCs. Our study is to explore the extracted primary stromal cells Q-Z1(OEMs), Q-Y19(OEMs), X-Y19 (AMs) and compared with the control ThESC. It was found that the expression level of LASP1 was relatively consistent with miR-218 in ESCs.

Many studies have confirmed that LASP1 is a downstream target gene of miRNA, and LASP1 is involved in the pathophysiological process of a variety of malignant tumors. The "eutopic endometrium determinism" of EMs, that is, the abnormal phenotype of the eutopic endometrium is determined by the specific genotype of the eutopic endometrium. Therefore, in recent years, many researches have been carried out on the specific genotype of the eutopic endometrium.. The study found that compared with the control group, the expression level of miRNA, and even the expression level of some proteins, in the endometrial tissue of EMs patients did have certain differences.

Through our qPCR and WB data, it was found that LASP1 in AMs and EMs was highly expressed in the selected original ESCs, while miR-218 was expressed at a low level. Besieds, the expression level of miR-218 and LASP1 expression were negative correlation. The results obtained verify our conjecture that LASP1 is highly expressed in EMs, and the mechanism is that miR-218 binds to the 3'UTR region of

LASP1 and negatively targets and regulates LASP1, which are consistent with the results of dual luciferase reporter gene assay. After transfection according to the above transfection principles, select conditional cells with high transfection efficiency at a transfection concentration of 50nM. Through the EdU proliferation experiment, it was found that the proliferation function of control ESCs and EMs resident ESCs were are consistent with the results of Qiu Yu et al, that is, miR-218 does not affect the proliferation ability of ESCs. And in this case, low expression of miR-218 enhanced the migration ability of ESCs. Conversely, high expression of miR-218 reduced the migration ability of ESCs. This result is consistent with the results of other studies on the function of LASP1 on cell migration.

Studies have reported that miR-218 can inhibit the proliferation of cervical cancer SCC cell lines, regardless of whether its HPV expression is positive or negative; the results also show that miR-218 does have an inhibitory effect on cervical cancer(Yamamoto et al., 2013). This study found that miR-218 has no proliferation effect on ESCs. Considering that although EMs have the characteristics of malignant tumors, they are still benign diseases and may not have the characteristics of continuous proliferation of malignant tumors. And this result also ruled out that the enhanced effect of miR-218 on the migration of endometrial stromal cells may be caused by cell proliferation, making the result of cell migration function more credible.

Based on the comprehensive analysis of all experimental results in this experiment, it can be inferred that in patients with EMs, the increased expression of LASP1 protein in the resident ESCs promotes the migration of ESCs. According to the "endometrial determinism" of EMs hotspot. ESCs have a specific phenotype, and then according to the pathogenesis of EMs, "menstrual blood reflux" and many other theories, the process of EMs formation is inferred. The specific genotypes of ESCs When its migration ability is enhanced, the intimal fragments containing ESCs enter the abdominal cavity with the menstrual blood reflux, and are planted in the ectopic lesions of the abdominal cavity in a relatively adapted environment, thereby forming ectopic lesions of EMs, which promotes the occurrence and development of EMs. This also explains why most of the menstrual blood reflux did not cause EMs, and combined with the multiple existing mechanisms of EMs, this result serves as a supplementary explanation and makes the mechanism of occurrence and development of EMs more clear.

For the detection of tissue immunofluorescence, it is interesting that the differential expression of miR-218 does not appear in the endometrium (including mesenchymal and epithelial cells). However, there are obvious signals around the endometrium (Figure.3), indicating that miR-218 may be transferred to the microenvironment (the microenvironment may be also changed) through exosomes secreted from blood vessels, and further act on the endometrium, changing the physiological state of the endometrium(Phinney & Pittenger, 2017; Yu, Odenthal, & Fries, 2016). This transmission signal is a paracrine signal from secretory cell to the target cell, therefore, miR-218 is already hard to detect. It may not be differentially expressed in the endometrium by immunofluorescence experiment, but in our qPCR results (Figure.1). We need to conduct further research on the exosomes in the blood of EMs patients, explore the differential expression of microRNA among them, and carry out subsequent studies.

Besides, we also found that miR-218 play a role in EMT. To verify the above results, we first ruled out the interference of antibodies (Vimentin and E-cadherin). We used the above two antibodies for immunohistochemistry in stromal and epithelial cells, and found that E-cadherin is specifically expressed on the membrane of epithelial cells, while Vimentin is specifically expressed in stromal cells (sFig 1), indicating that the expression pattern of these two antibodies is correct. Then, in ectopic endometrial tissue, both E-cadherin and Vimentin were significantly higher in epithelial cells than in ectopic stromal cells (R3 vs. R2) (Figure.5), indicating that E-cadherin and Vimentin are specific markers for EMs epithelial cells. The above data demonstrate that ectopic endometrial epithelial cells have a tendency to transform into ectopic stromal cells when miR-218 is inhibited. In addition, E-cadherin and Vimentin expression was higher in eutopic endometrial epithelial cells than in normal human eutopic endometrium (Figure.4). These results were consistent with the results in ectopic endometrium of EMs patients (Figure.4).

Conclusions

Our findings revealed that the aberrant inactivation of non-endometrial (endometriosis microenvironment) miR-218-5p plays an important role in the pathogenesis of adenomyosis through promoting the transition of ectopic endometrial stromal cells to epithelial cells by inhibiting LASP1.

Abbreviations

LASP1: LIM and SH3 protein1

EMs: Endometriosis

Ads: Adenomyosis

3'UTR: 3'untranslated region

EMT: Epithelial-mesenchymal transition

ESCs: Endometrial stromal cells

CEu: Control eutopic endometrium

AEu: Adenomyosis eutopic endometrium

AEc: Adenomyosis ectopic endometrium

EmL: Endometrial layer

MCL: Muscle layer

Declarations

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Authors' contributions

O.P.H, L.Q.W and Y.Z were responsible for the experimental design and drafting the manuscript, Z.Y.Z, L.Q.Z, Y.Q.W and K.H.Y were involved in carrying out the experiments, Z.Y.Z and Y.Q.W were responsible for analyzing and interpreting the data, F.Y.L, Y.L and B.C.Y were involved in the acquisition of tissues.

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Ethical Approval and Consent to participate

The patients signed a written informed consent form prior to recruitment. This study is in line with the Helsinki Declaration and approved by the Ethics Review Body Committee of the Jiangxi Maternal and Child Health Hospital.

Consent for publication

Not applicable.

Availability of supporting data

All data supported the conclusions during this study are included within the article.

Competing interests

There are no competing interests to declare.

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Figures

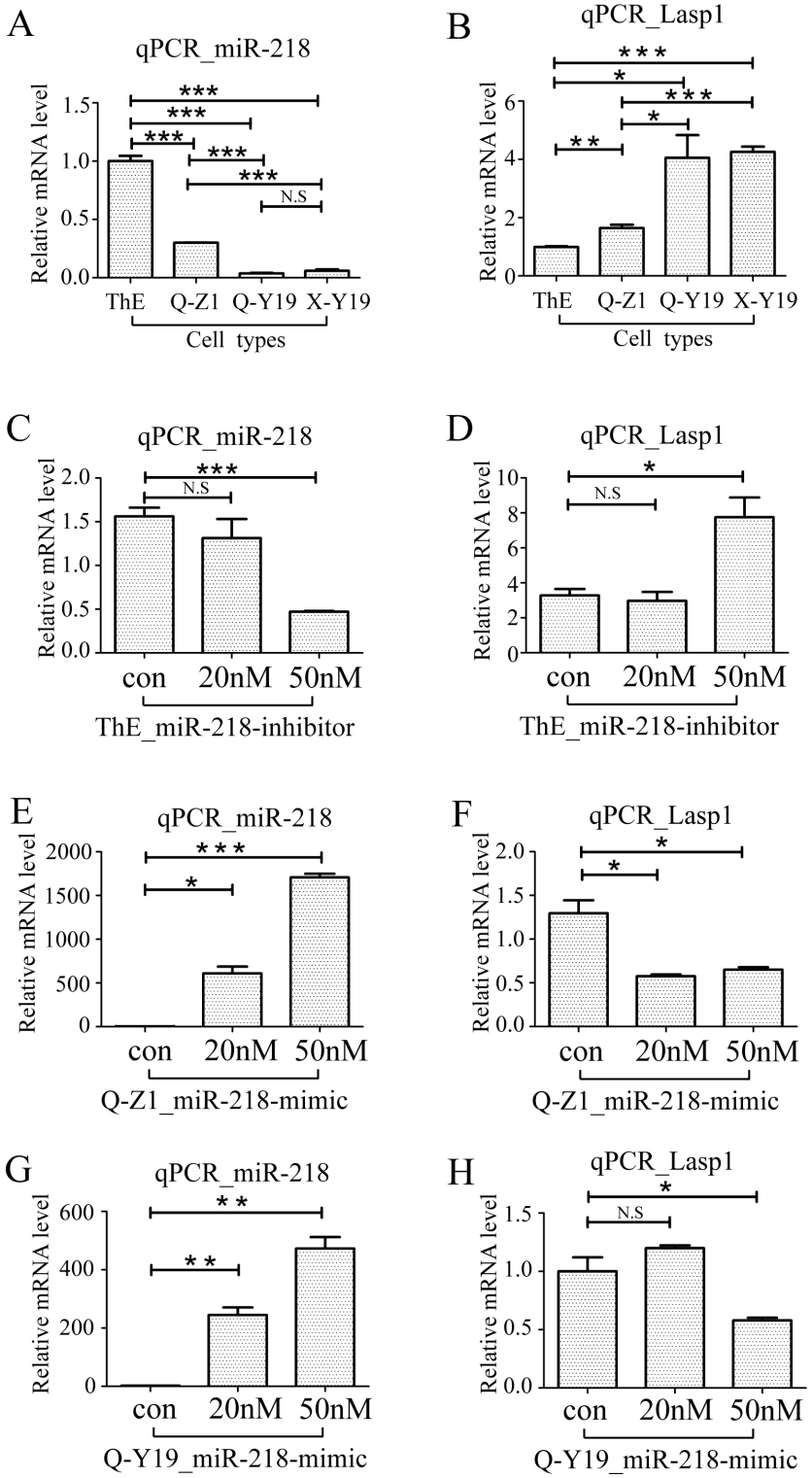

Figure 1

miR-218-5p inhibits LASP1 mRNA level in ESCs. (A) and (B) qPCR analysis of miR-218-5p and LASP1 mRNA levels in different ESCs, respectively. ThE: Eutopic stromal cell line of the control endometrium. Q-Z1: Primary eutopic stromal cell of the endometriosis endometrium. Q-Y19: Primary ectopic stromal cell of the endometriosis endometrium. X-Y19: Primary ectopic stromal cell of the adenomyosis endometrium. (C) and (D) qPCR analysis of miR-218-5p and LASP1 mRNA levels in ThESC transfected

with the control inhibitor, 20nM and 50nM miR-218-5p inhibitor respectively. (E) and (F) qPCR analysis of miR-218-5p and LASP1 mRNA levels in Q-Z1 transfected with the control inhibitor, 20nM and 50nM miR-218-5p inhibitor respectively. (G) and (H) qPCR analysis of miR-218-5p and LASP1 mRNA levels in Q-Y19 transfected with the control inhibitor, 20nM and 50nM miR-218-5p inhibitor respectively. P-values were determined by Student's t-test, *p<0.05, **p<0.01, ***p<0.001.

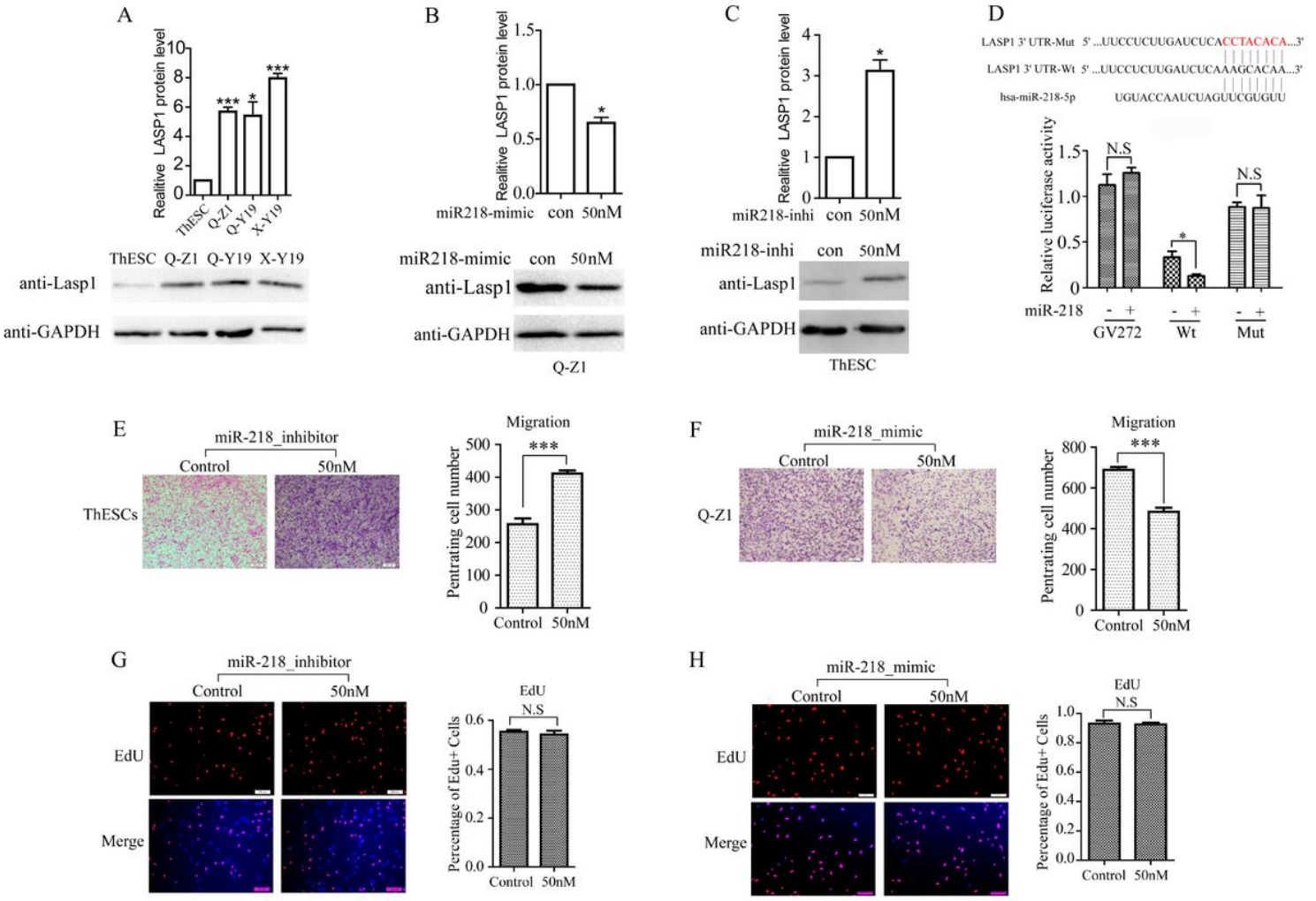

Figure 2

miR-218-5p has no effect on the proliferation of ESCs but can inhibit the migration of ESCs. (A) Western blot analysis and quantification of LASP1 protein level in lysates of different ESCs. (B) Western blot analysis and quantification of LASP1 protein level in lysates of Q-Z1 with or without 50nM miR-218-5p mimic. (C) Western blot analysis and quantification of LASP1 protein level in lysates of ThESc with or without 50nM miR-218-5p inhibitor. GAPDH was used as the loading control. P-values were determined by Student's t-test, *p<0.05, ***p<0.001. (D) Predicted miR-218-5p target recognition sites in the 3'-UTR of human LASP1. Mutation in the miR-218-5p target site is red. 3'UTR of LASP1 luciferase constructs, as indicated, were transfected into 293T cells together with indicated plasmid for 48h and subjected to a luciferase reporter assay. The results were normalized to the Renilla luciferase activity and are expressed as the fold change in relative luciferase activity compared with the control. (E) ThE cells were transfected

with control inhibitor or miR-218-5p inhibitor. (F) Q-Z1 cells were transfected with control mimic or miR-218-5p mimic. After 24 hours of transfection, cells were starved for 24h before cell migration assay was performed without matrigel transwell filters, Scale bar=200 μm. The migrated cells were stained and counted. Quantification was done in and is shown with counting six nonoverlaping fields. (G) and (H) EdU incorporation assays of ThE or Q-Z1 cells were transiently transfected with control inhibitor/miR-218-5p inhibitor or control mimic/miR-218-5p mimic, DAPI staining was included to visualize the cell nucleus (Blue), Scale bar=100 μm. Each bar indicates mean±SEM. of a representative experiment performed in triplicate. P-values were determined by Student's t-test. *** p<0.001.

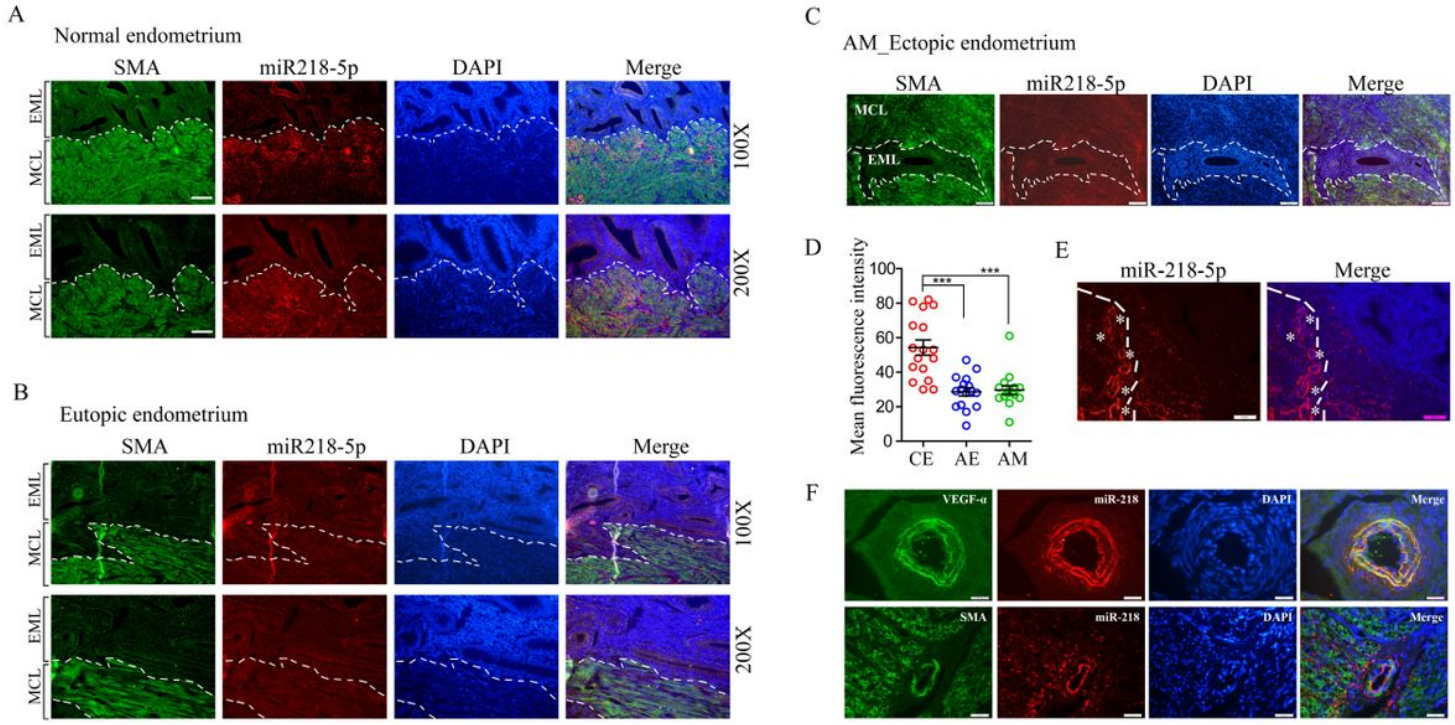

Figure 3

miR-218-5p was overexpressed in myometrium close to the endometrium of CEu. Immunofluorescent analysis of miR-218-5p (Red) and SMA(Green) in the (A) control eutopic endometrium (CEu), (B) adenomyosis eutopic endometrium(AEu) and (C) adenomyosis ectopic endometrium(AEc) respectively. EmL: endometrial layer, *means myometrium, DAPI staining was included to visualize the cell nucleus (Blue), Scale bar=100 μm. (D) Statistic analysis of Immunofluorescent staining of miR-218-5p in the myometrium of CEu (n=17), AEu (n=16) and AEc (n=16). P-values were determined by Student's t-test, ***p < 0.001. (E) Immunofluorescent analysis of miR-218-5p (Red) in the control endometrium tissue. (F) Immunofluorescent analysis of miR-218-5p (Red), VEGF (Green) and SMA (Green) in the control endometrium tissue. DAPI staining was included to visualize the cell nucleus (Blue), Scale bar=50 μm.

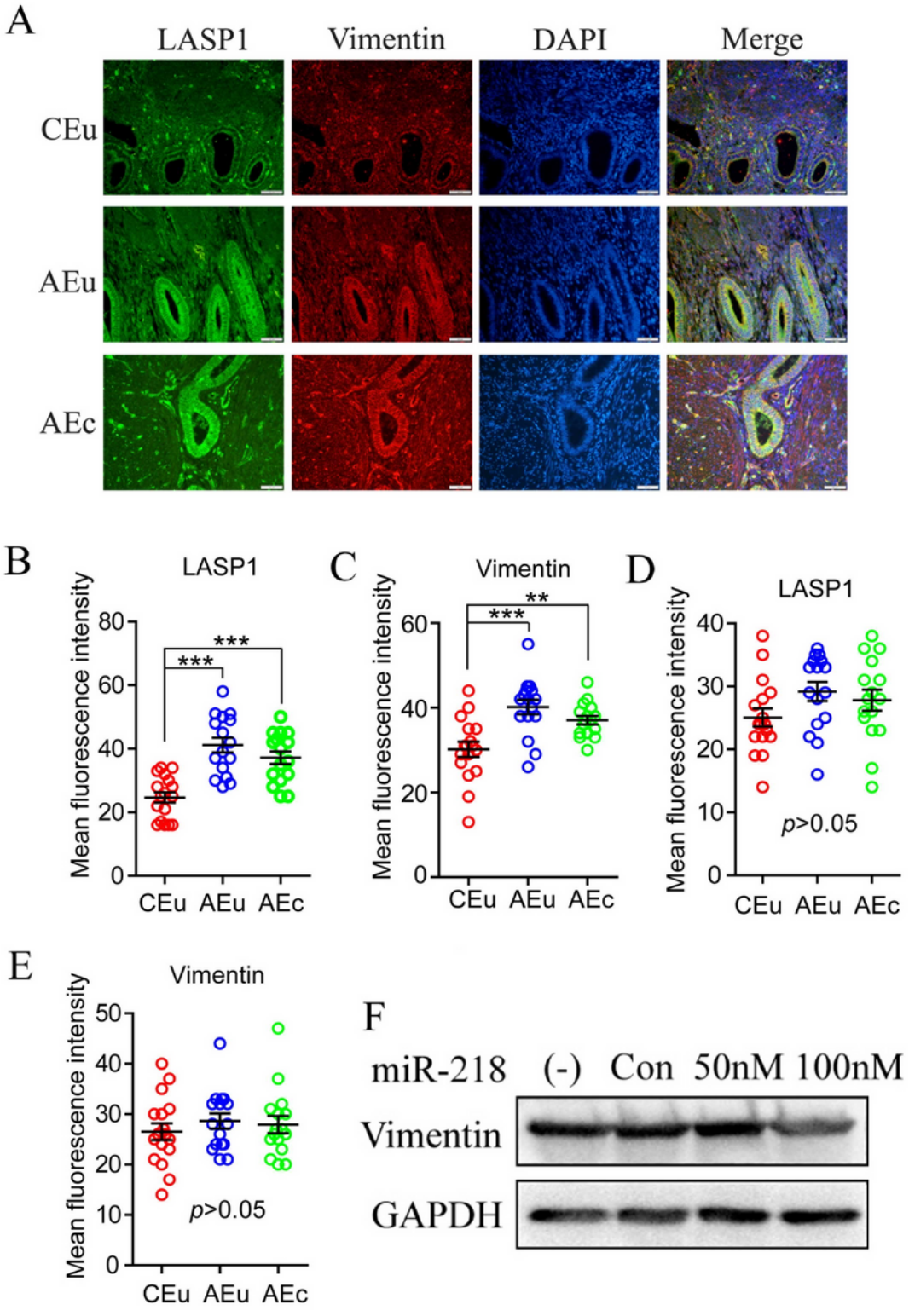

Figure 4

Inhibition of 218 prevents the epithelial-mesenchymal transition. (A) Immunofluorescent analysis of Vimentin (Red) and LASP1 (Green) in the control eutopic endometrium (CEu), adenomyosis eutopic endometrium(AEu) and adenomyosis ectopic endometrium(AEc) respectively. DAPI staining was included to visualize the cell nucleus (Blue), Scale bar=100 μm. (B) and (C) Statistic analysis of Immunofluorescent staining of LASP1 and VEGF-α in the gland epithelial cell of three groups. (D) and (E)

Statistic analysis of Immunofluorescent staining of LASP1 and VEGF-α in the stromal cell of three groups: CEu (n=17), AEu (n=16) and AEc (n=16). P-values were determined by Student's t-test, ***p < 0.001. (F) Western blot analysis showing effects of miR-218-5p on protein levels of Vimentin. GAPDH was used as the loading control.

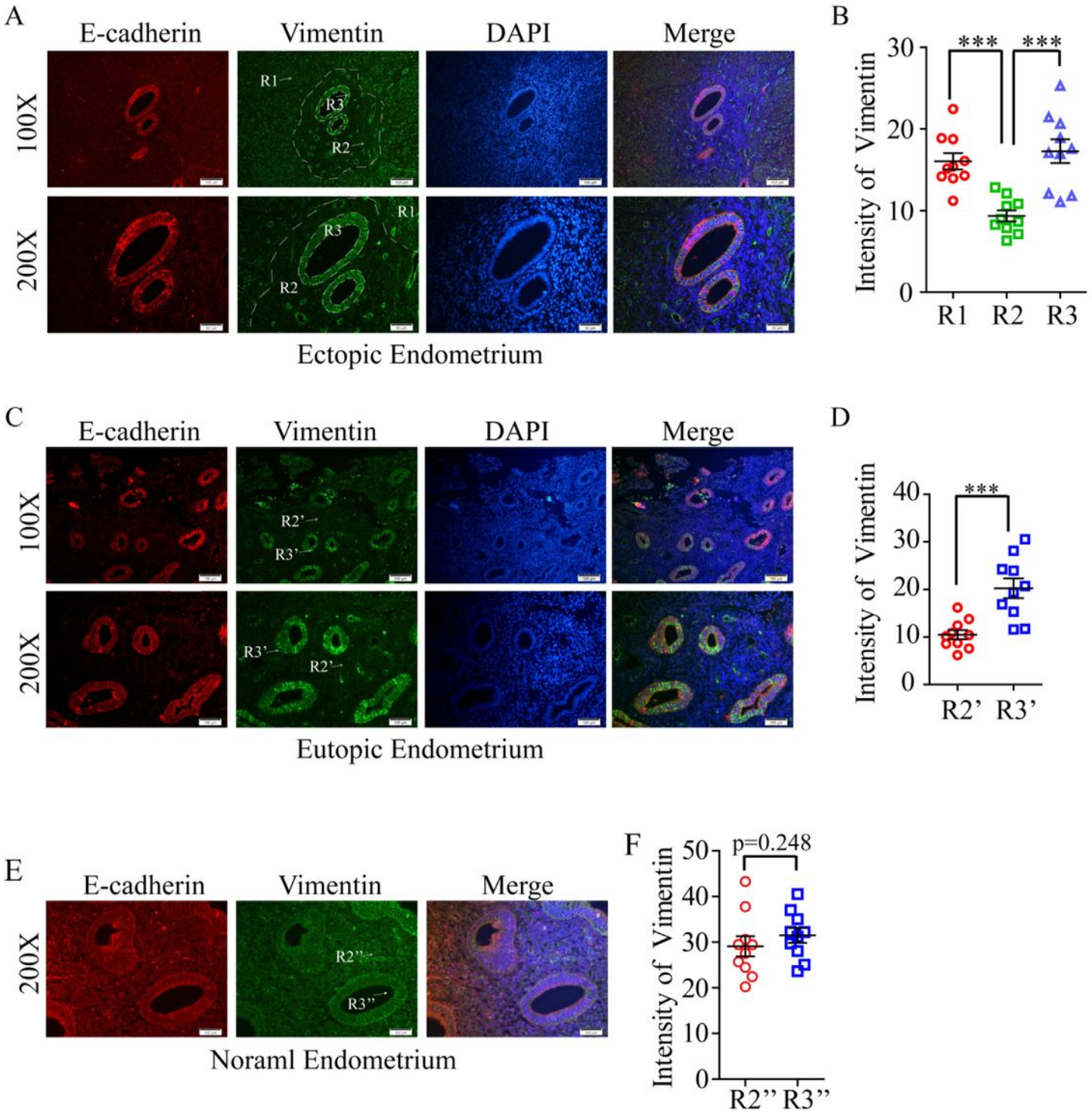

Figure 5

Both E-cadherin and Vimentin were high expression levels in EMs epithelial cells. (A) Immunofluorescent analysis of E-cadherin (Red) and Vimentin (Green) in the ectopic endometrium of EMs tissue. (B) Statistic analysis of Immunofluorescent staining of Vimentin in the region of R1, R2 and R3 EMs tissue(n=10). (C) Immunofluorescent analysis of E-cadherin (Red) and Vimentin (Green) in the eutopic endometrium of EMs tissue. (D) Statistic analysis of Immunofluorescent staining of Vimentin in the region of R2' and R3' from EMs tissue (n=10). Scale bar = 100 μm for upper picture and 200 μm for below picture. DAPI was used to stain nuclei. P-values were determined by Student's t-test, ***p < 0.001. (E) Immunofluorescent analysis of E-cadherin(Red) and Vimentin(Green) in the normal endometrium tissue. (F) Statistic analysis of Immunofluorescent staining of Vimentin in the region of R2" and R3" from normal endometrium tissue(n=10). Scale bar = 50 μm. DAPI was used to stain nuclei. P-values were determined by Student's t-test.

Supplementary Files

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