

Hsa_circ_0063526 promotes the development of endometriosis by sponging miRNA-141-5p

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Research

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Abstract

Background:

Endometriosis can cause various social burdens. Abnormal circular RNAs level lead to changes of related gene expression, thereby mediating the occurrence and development of a series of diseases. The research of circRNA in endometriosis is still in its infancy. This study will explore the role of hsa_circ_0063526 with microRNA-141-5p in the development of endometriosis.

Methods:

The expression of genes was detected by RT-qPCR. Transwell, wound-healing, EdU assay were performed on endometriosis patient's End1 / E6E7 cell. PCR and immunohistochemistry were used to detect the expression of candidate regulatory genes in ectopic lesions in endometriosis mice model.

Results:

The expression level of hsa_circ_0063526 in endometriosis patients' ectopic tissue is higher than control ($P < 0.05$), The expression levels of hsa_circ_0063526 and miRNA-141-5P in ectopic tissue of endometriosis were negatively correlated ($P < 0.05$).

Knockdown hsa_circ_0063526 inhibited the invasion, metastasis, and proliferation ability of End1 / E6E7 cell. The inhibition of microRNA-141-5p can rescue this inhibitory effect ($P < 0.05$). In-vivo experiments showed miR-141-5p and si-hsa_circ_0063526 treatment reduced the lesion size and regulated endometriosis genes.

Conclusion:

Hsa_circ_0063526 is probably involved in regulating the occurrence and development of endometriosis by sponging miR-141-5p. hsa_circ_0063526 and miR-141-5p are possible to become biomarkers and therapeutic targets for endometriosis.

Research In Context

Evidence before this study

Circular RNAs play essential roles in many diseases. The research of circRNA in endometriosis is still in its infancy. In our previous study, we did microarray circRNAs expression detection and RT-qPCR verification in endometriosis vs control, we found in the endometriosis group, hsa_circ_0063526 (circ-RanGAP1) expression level is higher. Although the important biological functions of circRNAs have been gradually explored, their underlying mechanism in endometriosis remains unclear.

Added-value of this study

In the present study, we found that the hsa_circ_0063526 expression in endometriosis patients were different from that of the control group. Hsa_circ_0063526 may contribute to the occurrence and development of endometriosis by sponging miR-141-5p.

Implications of all the available evidence

Hsa_circ_0063526 is probably involved in regulating the occurrence and development of endometriosis by inhibiting miR-141-5p through sponging. hsa_circ_0063526 and miR-141-5p are possible to become biomarkers and therapeutic targets for endometriosis.

Introduction

Endometriosis is a common estrogen-dependent chronic disease, affecting about 10% of childbearing-aged women, and 20–50% of the infertile patients were combined with endometriosis, the disease causes infertility, and further malignant transformation (1). The etiology and pathogenesis of endometriosis are not clear. Sampson first proposed the theory of menstrual blood reflux, the endometrial glandular epithelium and mesenchymal cells flow back into the fallopian tube and the abdominal cavity with menstrual blood, implanted in the basin of abdominal organs such as the ovaries or pelvic peritoneum. The cells continue to grow there, so form the endometriosis(2). Although endometriosis is a benign disease with benign histomorphology, its clinical behaviors show similar characteristics of malignant tumors' invasion, metastasis, and implantation, so it is also called "benign cancer". Lang proposed the "Eutopic endometrium determinism" of endometriosis, that is, the adhesion, invasion, and growth of endometrium tissue in "ectopic" are determined by the property of the endometrium itself, and the menstrual blood reflux is only a potential auxiliary pathway(2). Recently, the pathogenesis of endometriosis increasingly attributes to a variety of genes and factors closely related to genetics (3). Treatment for endometriosis has many side effects, including stopping the menstrual cycle during medication, etc(4–7). New diagnostic indicators and non-hormonal therapy for endometriosis are urgently needed.

MicroRNAs are highly conserved and considered important post-transcriptional regulators(8). At present, relevant studies on the interaction between miRNA and endometriosis are gradually attracting attention. Many studies have regarded miRNA as a potential new biomarker for endometriosis (9–12).

In our research team, microRNA solexa sequencing and RT-qPCR verification were used to test the serum samples of endometriosis patients in the early stage(13–16), the result showed the expression level of miR-141 was lower than control (17). Studies have demonstrated the down-regulation of the mir-200 family (Include miR-141-5p) in endometriosis patients' plasma(17).

Several studies have explored the use of miR-141-5p to treat a range of diseases. For example, Kim et al previously synthesized miR-141-5p hydrogel that shown effective results in liver cancer mice(18). Rekker et al reported that in the serum of endometriosis patients, the expression of circulating microRNA-200s family including miR-141-5p was lower than control(11). Liang et al. used miR-200c to suppress

endometriosis in cells and a mice model(19). Members of our research team also reported that the reduction of miR-141-5p in endometriosis tissue promotes the development of endometriosis mainly by regulating EMT(epithelial-mesenchymal transformation) (20).

CircRNAs exist in eukaryotic cells as covalently closed rings, without 5 'or 3' polarity or polyadenylate, and are more conserved and stable than linear RNAs. It used thought to be a rare type of RNA, just "waste products" from the process of RNA cutting. More and more evidence showed that circRNAs are involved in the proliferation, invasion, and metastasis of various diseases(21–24). CircRNAs regulate gene expression levels mainly by binding to proteins or sponging microRNAs(25). These molecular interactions will open up new prospects for the diagnosis and treatment of endometriosis. However, the pattern and potential role of circRNAs in the tissue of patients with endometriosis have not been elucidated(20, 24, 26).

In our previous study, we did microarray circRNAs expression detection and RT-qPCR verification in endometriosis versus control, we found in the endometriosis group, hsa_circ_0063526 (circ-RanGAP1) expression level is higher, bioinformatics analysis revealed that circ-RanGAP1 and miRNA-141-5p have complementary binding sites(27). Furthermore, we hypothesized that circRNAs may involve in the development of endometriosis. We aim to explore the correlation between hsa_circ_0063526 with miR-141-5p and endometriosis.

Methods

2.1 Materials

2.1.1 Clinical specimens

Tissue samples were collected from child-bearing-aged women who underwent surgery from February 2019 to January 2020 in the department of gynecology of The Second Xiangya Hospital. Fresh proliferation stage endometriosis cyst samples and endometrial tissue were snap-frozen and stored in a liquid nitrogen container. The study was approved by the ethics committee of The Second Xiangya Hospital. Before the samples were taken, informed consent was assigned by each patient. The clinicopathological data of endometriosis patients, including age, histological subtype, clinical-stage, cyst size was retrieved (Supplementary Table 1–1).

Experimental group: The ectopic endometriosis group of EMs patients, a total of 31 patients, all of whom were diagnosed with ovarian endometriosis by laparoscopy plus histopathology. The endometriosis lesions were collected intraoperatively as the ectopic endometrium group (American Fertility Society stage III-IV).

Control group: 10 patients with secondary infertility caused by fallopian tube obstruction factors confirmed by the combined operation of uterus and abdomen. The pathological diagnosis is proliferation stage endometrium. The sample size was calculated by calculator on powerandsamplesize.com.

2.1.2 cell line

The human renal epithelial cell line HEK293T cells, End1/E6E7 endometrial cells from endometriosis patients were purchased from Bena Culture Collection (Beijing, China).

2. 1.3 plasmid

pmiR-RB-Report™ hsa_circ_0063526 (hsa_circ_0063526 3'UTR:1044–1103) -WT(hsa_circ_0063526-WT) □
pmiR-RB-Report™ hsa_circ_0063526 (hsa_circ_00635263'UTR:1044–1103) - MUT(1273-1280GGAAGAT >
CCTTCTA) □ hsa_circ_0063526-MUT □ plasmids are provided by Ribobio(Guangzhou, China).

2. 1.4 Primers used for RT-qPCR detection

All primers were verified by PubMed BLAST and previous articles. The primers used in the experiment are shown in Table 1.

Table 1
Primer information used in this paper

| Primers | Sequences of 5 'to 3' |
|--------------------------|------------------------------|
| ZO-1 Forward | GAATGATGGTTGGTATGGTGCG |
| ZO-1 Reverse | TCAGAAGTGTGTCTACTGTCCG |
| E-cadherin Forward | TCCATTTCTTGGTCTACGCC |
| E-cadherin Reverse | CACCTTCAGCCAACCTGTTT |
| N-cadherin Forward | GTGCCATTAGCCAAGGGAATTCAGC |
| N-cadherin Reverse | GCGTTCCTGTTCCACTCATAGGAGG |
| Vimentin Forward | AGCCGAAAACACCCTGCAAT |
| Vimentin Reverse | CGTTCAAGGTCAAGACGTGC |
| K-ras Forward | AGACACAAAACAGGCTCAGGA |
| K-ras Reverse | TTCACACAGCCAGGAGTCTTT |
| Beta-actin Forward | GGGGTGTGGAAGGTCTCAA |
| Beta-actin Reverse | GGCATCCTCACCTGAAGTA |
| ER-alpha Forward | AAGAGCTGCCAGGCCTGCC |
| ER-alpha Reverse | TTGGCAGCTCTCATGTCTCC |
| ER-beta Forward | GCTCAATTCCAGTATGTACC |
| ER-beta Reverse | GGACCACATTTTTGCACT |
| IL-6 Forward | GTCAACTCCATCTGCCCTTCAG |
| IL-6 Reverse | GGTCTGTTGTGGGTGGTATCCT |
| ZEB1 Forward | TGAATCATCGCTACTCCTACTGT |
| ZEB1 Reverse | TTTCACTGTCTTCATCCTCTTCCC |
| Notch-1 Forward | GTCAACGCCGTAGATGACC |
| Notch-1 Reverse | GTCAACGCCGTAGATGACC |
| MAPK14 Forward | GAACAAGACAATCTGGGAGGTG |
| MAPK14 Reverse | TTCGCATGAATGATGGACTGAA |
| GAPDH Forward | GCACCGTCAAGGCTGAGAAC |
| GAPDH Reverse | TGGTGAAGACGCCAGTGGA |
| hsa_circ_0063526 Forward | AGATTCTGGACCCTAACACTGG |

| Primers | Sequences of 5 'to 3' |
|------------------------------------|---|
| hsa_circ_0063526 Reverse | CTCTTGCCTTTGAAACTCAGCT |
| miR-141-5p Forward | CGCGCATCTTCCAGTACAGT |
| miR-141-5p Reverse | AGTGCAGGGTCCGAGGTATT |
| miR - 141-5p reverse transcription | GTCGTATCCAGTGCAGGGTCCGAGGTA- TTCGCACTGGATACGACTCCAAC |

2. 1.5 Design and synthesis of si-hsa_circ_0063526 and miR-141-5p inhibitor

For the sequence of the target gene hsa_circ_0063526, siRNA sequence and random independent short RNA sequence (NC-RNA) as the control were designed and synthesized by Genepharma (Suzhou, China):

si-hsa_circ_0063526-1

Sense: 5' - GaccuaaacacugggucugTT-3'

Antisense: 5' - cagAcccaguguuagGGUCTT-3'

si-hsa_circ_0063526-2

Sense: 5' - AacacugggucugCagauctt-3'

Antisense: 5' - GaucugcagAcccaguguutt-3'

si-hsa_circ_0063526-3

Sense: 5' -cacugggucugCagaucuctt-3'

Antisense: 5' -GagaucugcagACCcagugTT-3'

Inhibitor-miR-141-5p: a short-stranded RNA modified by the complementary sequence of miR-141-5p, provided by Ribobio(Guangzhou, China). MicroRNA-141-5p sequence is as follows: microRNA-141-5p: 5'-UAacaccugucugGuaaagaugg-3' .

2.2 RNA extraction and RT-qPCR

The RNA was extracted by Trizol reagent (Beijing Dingguo Biological Technology Co., Ltd.). Total RNA was reversely transcribed into cDNA and real-time-qPCR analysis was performed using Universal SYBR Green MasterMix Kit (Vazyme, China). $2^{-\Delta\Delta Ct}$ method was used for calculating the relative RNA expression.

2.3 Bioinformatics analysis of hsa_circ_0063526 complementary miRNAs

Miranda, TargetScan, RNA22 v2, RNAhybrid software was used to predict the potential target miRNAs and the target genes of the miRNAs involved. The miRNA complementing hsa_circ_0063526 was predicted, and further research and verification were conducted.

2.4 Dual-luciferase reporter assays

The recombinant plasmid of double luciferase reporter hsa_circ_0063526-WT and hsa_circ_0063526-MUT was designed and synthesized according to the complementary pairing sequence of miR-141-5p and hsa_circ_0063526-MUT. 293T cells were seeded with 1.0×10^4 cells per well. The miRNA mimics or non-target Control vector and target gene 3' UTR double reporter vector or mutant vector were diluted in 5 μ L Opti-MEM medium, and the transfection reagent was diluted with 0.25 μ L Lipo6000 in 5 μ L Opti-MEM medium. Before plasmids and Mimics were added to the cells, a 90 μ L culture medium was added to each well. Mimics transfection concentration was 50nM and plasmid concentration was 50ng/ well. Each group was set with 3 replicates. After 48h of transfection, the medium was extracted and added with PBS. Luciferase reagent was added and shaken for 10min. The medium was transferred to LUMITRAC™ 200 96 well white cell culture plate for fluorescence determination. Finally, 30 μ L Stop reagent was added to each well for spectrophotometric determination.

In Sect. 2.5-2.8, End1/E6E7 cells were divided into five groups, specifically:

- 1) si-hsa_circ_0063526 group (si-hsa_circ_0063526 transfection group): The si-hsa_circ_0063526 siRNA was transfected to knockdown hsa_circ_0063526.
- 2) si-hsa_circ_0063526 + inhibitor-miR-141-5p group (si-hsa_circ_0063526 + inhibitor-miR-141-5p group): the si-hsa_circ_0063526 and miR-141-5p were transfected, knockdown of hsa_circ_0063526 siRNA and inhibition of miRNA-141-5p performed at the same time.
- 3) Inhibitor of miR-141-5p group (inhibitor of miR-141-5p transfection group): Transfected the inhibitor of miR-141-5p to inhibit the function of miR-141-5p.
- 4) NC-RNA group (NC-RNA transfection group): group transfected with random short RNA sequences.
- 5) Blank control group: transfection reagent and RNA were not added.

2.5 Wound healing assays

To detect the migration of cells, we conducted wound healing assays in vitro. Sterile 100 μ L Tips were used to draw on the confluent monolayer cells in the cell culture Wells. Washed with PBS and put the 6 well plates back into the CO₂ at 37°C for 48 hours. The cells were observed under an inverted microscope. The width of the scratch was measured and recorded using ImageJ software.

2.6 Transwell assays

To detect the invasion ability of each group, a Transwell assay was performed in vitro. Matrigel was transferred from -20°C to a 4°C and thawed for 12 hours. The Matrigel was diluted without serum. An upper chamber with a diameter of $8\ \mu\text{m}$ was used. The Matrigel was carefully placed into the upper chamber. The Transwell plate was placed back into the CO_2 incubator at 37°C and incubated for 24 hours. 1×10^4 of End1/E6E7 cells in $200\ \mu\text{l}$ serum-free cell suspension was added to the upper chamber. $600\ \mu\text{L}$ high-sugar DMEM medium containing 10% fetal bovine serum was added into the lower chamber. Put the Transwell plate back to CO_2 at 37°C and incubated for 36 hours. Then the cells were stained with 0.5% crystal violet. The results were analyzed and counted with ImageJ software.

2.7 EdU assays

EdU (5-acetylene 2' -deoxyuridine) proliferation assay was performed using the EdU Apollo567 proliferation in vitro kit (RiboBio, Guangzhou, China).

End1/E6E7 cells (2×10^4) were seeded to a 96-well plate. The cells were transfected with si-hsa_circ_0063526, miR-141-5p inhibitor, si-circ_0063526 + miR-141-5p inhibitor, and control siRNA. After 48 hours, $100\ \mu\text{L}$ diluted 50 M EdU was added to each 96-well plate. Then the cells were fixed and dyed with 1X Apollo staining solution and DAPI. Observed and be taken pictures immediately. The number of EdU positive cells and the total number of cells were calculated. The results were analyzed and counted with ImageJ software.

2.8 Enzyme-linked immunosorbent assay (ELISA)

Human E-Cadherin ELISA kit (R&D Systems, MN, USA) was used for E-Cadherin expression detection. $100\ \mu\text{L}$ cell culture supernatants of five groups were first added to the wells of the 96-well ELISA plate. standard substance was set up in concentrations of 0, 0.31, 0.63, 1.25, 2.50, 5.00, 10.00, 20.00 ng/ml. The enzyme-labeled plates were incubated on a micro-oscillator with a speed of 700rpm for 2 h, washed 4 times. Next, $200\ \mu\text{L}$ enzyme marker E-cad conjugate was added into each well. Finally, substrate solution and termination solution were added to each well. The OD value at 450 nm was determined within 30 min. Taking OD value of E-cad standard as ordinate (taking the average value of the concentration of three pores) and dilution concentration of a standard substance as abscissa, the standard curve was drawn, and the E-cad contents of the samples were calculated according to the standard curve.

2.9 Induction of endometriosis in mice

Endometrium was obtained from 18 childbearing-aged women who underwent hysterectomy for uterine myoma at The Second Xiangya Hospital. According to Noyes et al. criteria (1975), the menstrual cycle was histologically confirmed as the proliferative phase. The patients were not received hormone therapy for at least 6 months. All experimental protocols were approved by The Ethics Committee under the declaration of Helsinki, and all women signed informed consent. The endometrium is obtained by an

endometrial specimen collector (J-ES-090500; Cook, USA) and cut into 2 mm diameter fragments and incubated in DMEM, PEN/STREP (Changsha, China) culture medium at 4°C before implantation. Female nude mice aged 5 to 6 weeks were purchased and reared in the Department of Laboratory Animals in Central South University. They maintained five animals in each cage during a 12-hour light and 12-hour dark cycle (8 a.m. to 8 p.m.) in a barrier system.

The modified endometriosis model previously used in our laboratory and widely by previous scholars was used to induce endometriosis in 30 mice. According to this protocol, endometrial tissue fragments of patients of the same size (2 mm) were sutured to the peritoneal surface of each mice. Mice were anesthetized by sodium pentobarbital and were laparotomies through a midline incision. Two pieces of endometrial tissue fragments were sutured by 5 - 0 polyglactin suture on the surface of the left and right abdominal wall respectively. Then, the peritoneum and skin were closed.

2.10 microRNA-141-5p-agomir and si-hsa_circ_0063526 agomir treatment

Thirty experimental endometriosis animals were randomly allocated into six groups of 5 in each. 5 days after induction of endometriosis, miRNA-141-5p therapy began with miRNA-141-5p-agomir (uaacacugucugguaaagaugg Genepharma Company, China) + vector or scramble miRNA-agomir + vector or only saline as two control group by another researcher. si-hsa_circ_0063526 agomir therapy began with siRNA-hsa_circ_0063526 agomir (sense: 5'-CACUGGGUCUGCAGAUCUCTT-3' Antisense: 5'-GAGAUCUGCAGACCCAGUGTT-3' Genepharma Company, China) + vector or scramble agomir + vector or only saline as two control group. RNAs were intraperitoneally injected into the mice by transfection vector Entranster-R4000 carrier (Engreen Biosystem, China). An Entranster-R4000 + RNA mimic mixture was prepared. Each injection of 0.5 ml 5% dextrose mixture includes 90 µg oligonucleotides and 20 µL of transfection vector. The mice were injected intraperitoneally every 3 days for 2 weeks.

2.11 Evaluation of lesions

After microRNA-141-5p-agomir and si-hsa_circ_0063526-agomir treatment for 2 weeks, the animals were cervical dislocated, and the endometriosis lesions were collected from the abdominal cavity of mice. The volume of lesions was calculated by the formula of minimum diameter² * maximum diameter/2. Left abdominal lesions were preserved in RNA stabilized solution (Qiagen, Germany) for extraction of mRNA, RT-qPCR was used to detect gene expression, and the right abdominal lesions were preserved in 4% polyformaldehyde solution for immunohistochemical study. Endometriosis was observed under a light microscope after H & E staining by a third researcher who was concealed from the group allocation. Image J (NIH, USA) was used for the calculation of the lesion area.

2.12 Immunohistochemistry

We used 4% paraformaldehyde to fix lesions and paraffin to embed lesions. Tissue was cut into slices about 5 microns and fixed on glass slides, then boiled in sodium citrate (pH = 6) solution in high pressure for 15 minutes to repair the antigen. 10% goat serum was used for antigen blocking. Slides were

incubated at 4°C overnight with anti-E-cadherin (1:1500; Proteintech, USA), anti-N-cadherin (1:1500; Proteintech, USA), anti-Vimentin (1:1500; Proteintech, USA) antibodies to determine protein expression. And dyed with DAB (Well-Biology, Changsha, China). Tissue sections were restained with hematoxylin (Well-Biology, Changsha, China). Stained section images were taken with OLYMPUS BX63 (OLYMPUS, Japan) and analyzed by Image-pro Plus 7.0.

2.13 Statistical analysis

All statistical analysis was performed in GraphPad Prism 7.0 software (Lahora, USA). All in vitro experiments were carried out three times. Data are mainly expressed as mean \pm standard deviation, mean \pm standard error (%), or count. T-test or Wilcoxon rank-sum test were used to compare the two groups. Kruskal-Wallis test or one-way ANOVA was used for comparison of three or more groups. Whether there is a correlation between hsa_circ_0063526 and the expression level of miR-141-5p was detected by Pearson correlation analysis. $P < 0.05$ was the standard for a statistically significant difference.

2.14 Role of the funding source

This study was funded by The National Natural Science Foundation of China (81671437,81771558) and Shenzhen People's Hospital. The sponsors helped with design, interpretation of data, and decide submission.

Results

3.1 Comparison of relative expression of hsa_circ_0063526 between endometriosis patients and the control group

RT-qPCR was used to analyze the expression level of hsa_circ_0063526 in the endometriosis group versus the control group. The results were shown in Fig. 1. Compared with the control group, the hsa_circ_0063526 level was higher in the endometriosis group. We found that the difference of means \pm SEM was 0.6856 ± 0.1735 ($P < 0.01$).

3.2 Bioinformatics analysis and Luciferase assay about hsa_circ_0063526 and miRNA-141-5p

We used bioinformatics analysis (starBase, www.starBase.sysu.edu.cn, RNAhybrid software) to predict the downstream target miRNA of hsa_circ_0063526 (Figure.2a). We performed a double luciferase reporter assay on the binding site (Fig. 2b&c). The double luciferase assay showed that the co-transfection of hsa_circ_0063526 wild-type and miR-141-5p mimic significantly reduced luciferase activity compared with the control group. Compared with the co-transfection of hsa_circ_0063526 mutant and miR-141-5p mimic, the reporter gene expression was rescued, and it indicated that miR-141-5p mimic could bind to hsa_circ_0063526.

3.3 correlation between miR-141-5p and hsa_circ_0063526 expression

RT-qPCR was used to determine the relative expression of miR-141-5p between endometriosis patients and the control group (Fig. 3a). We found the difference of means \pm SEM was 0.5994 ± 0.08425 ($P < 0.01$). Pearson correlation analysis of the expression levels of hsa_circ_0063526 and miR-141-5p in the lesion tissue of endometriosis showed a negative correlation ($r = -0.4267$, $p = 0.02$, Fig. 3b). Pearson correlation analysis further indicated the interaction between hsa_circ_0063526 and miR-141-5p.

Due to the overexpression of hsa_circ_0063526, we constructed different siRNAs to knockdown hsa_circ_0063526. To determine the knockdown efficiency, three fluorescent small interfering RNAs were designed and synthesized. Fluorescent images showed successful transfection of siRNA into End1/E6E7 cells. Results showed that interference sequence 3 most significantly reduced the expression of hsa_circ_0063526 (fig.s1).

After transfection with si-hsa_circ_0063526, the expression level of hsa_circ_0063526 in End1/E6E7 cells in the si-hsa_circ_0063526 group was significantly decreased ($P < 0.05$, Fig. 3c), and the expression level of miR-141-5p was significantly increased ($P < 0.05$, Fig. 3d), compared with that in the Blank group and the si-NC group. The results showed that hsa_circ_0063526 could negatively regulate the expression level of miR-141-5p in End1/E6E7 cells.

3.4 Down-regulation of hsa_circ_0063526 can inhibit the proliferation, invasion, and migration of endometriosis cells

Migration, invasion, and proliferation of ectopic endometrial cells are essential pathological processes for the development of endometriosis. Therefore, 72 hours after transfection of si-hsa_circ_0063526, this study further explored the changes of cell migration and invasion and cell proliferation ability after down-regulation of hsa_circ_0063526. First of all, the ability to invasion by Transwell experiments, after End1 / E6E7 cell transfected with hsa_circ_0063526 siRNA, the cells entering the lower chamber was relatively lesser compared to the control group, cell invasion ability decreased. Difference between means \pm SEM = 70.75 ± 10.99 ($P < 0.05$, Fig. 4). The ability of cell migration was tested by wound healing experiment, after End1 / E6E7 cell transfected hsa_circ_0063526 siRNA, the width of the cell scratch was wider than that of the control group. The ability of cell migration was decreased. Difference between means \pm SEM = -333.3 ± 72.12 ($P < 0.05$, Fig. 5). Further, the effect of down-regulation of hsa_circ_0063526 on cell proliferation was detected by using EdU cell proliferation assay. 72 h after siRNA transfection, compared with the si-NC control group, the EdU test results showed that the proportion of End1/E6E7 cells in the mitotic stage was lesser after the down-regulation of hsa_circ_0063526, and the proliferation capacity of the cells was decreased after siRNA transfection. Difference between means \pm SEM = 32.38 ± 6.639 ($P < 0.05$, Fig. 6). PCR and ELISA results showed that the expression of E-cadherin mRNA, an important epithelial marker of EMT, was up-regulated ($P < 0.05$, Fig. 7).

3.5 Down-regulation of miR-141-5p can rescue the proliferation, invasion, and migration of endometriosis inhibited by hsa_circ_0063526.

However, after co-transfected with si-hsa_circ_0063526 + inhibitor-miR-141-5p, the proliferation, invasion, and migration of endometriosis cells were rescued ($P < 0.05$, Fig. 4, 5, and 6). PCR and ELISA results showed that the expression of E-cadherin, an important epithelial marker of EMT, was down-regulated compared with the si-hsa_circ_0063526 group ($P < 0.05$, Fig. 7).

The above experimental results showed that knockdown hsa_circ_0063526 inhibited the development of endometriosis. Inhibition of microRNA-141-5p can rescue the inhibitory effect brought by knockdown of hsa_circ_0063526, that is to say, hsa_circ_0063526 promotes the development of endometriosis through sponging (inhibition) of microRNA-141-5p.

3.6 Comparison of miR-141-5p treatment and pathological changes

No adverse reactions were observed in mice treated with miR-141-5p. At the end of miRNA-141-5p treatment, mice were sacrificed by cervical dislocation and endometriosis lesions were collected. First, we evaluated the volume of the lesions between the miR-141-5p treatment group and the control group. All lesions were cystic. Lesions in miR-141-5p treatment groups were relatively small. (Figure 8 a,b,c)

Besides, the pathological difference of the endometrial part is further compared by the histological area. The histological area of all lesions was observed under the microscope to exclude muscle and destructed parts. The histological area of endometriosis in abdominal lesions of the miR-141-5p treatment group was significantly smaller than the two control groups ($p < 0.05$, Figure 8 d-g).

3.7 Expression difference of endometriosis-related genes

The differences in gene expression related to endometriosis were detected by RT-qPCR. The expression of some genes known to promote the development of endometriosis decreased in the miR-141-5p group. Expression of N-cadherin, Vimentin, K-ras, MAPK-14, ER- α , ER- β , ZEB-1 was decreased in the miR-141-5p treatment group ($P \leq 0.05$ Figure 9). The quantitative decrease in gene expression is 6.5-fold for K-Ras, 1.81-fold for MAPK14, 13.9 fold for ER- α , 97.2 fold for ER- β , 4.63 fold for N-Cadherin, 2.37 fold for Vimentin, 3.98-fold for ZEB-1 in 141 treated group compared to saline group. Expression levels of miR-141, E-Cadherin, ZO-1 (Zona Occludens 1) were increased in the miR-141-5p treatment group ($P \leq 0.05$, Figure 9). Expression levels of Notch, IL-6 was not statistically significant between treatment and control groups ($P \geq 0.05$, Figure 9).

Immunohistochemistry results showed that the protein expression level of the miR-141-5p treatment group was significantly changed. The intensity of N-cadherin and vimentin in stromal cells decreased in the miR-141-5p treatment group (Figure 10).

3.8 Comparison of si-hsa_circ_0063526 treatment and pathological changes

No adverse reactions were observed in mice treated with si-hsa_circ_0063526 agomir. At the end of si-hsa_circ_0063526 agomir treatment, mice were sacrificed by cervical dislocation and endometriosis lesions were collected. We evaluated the volume of the lesions between the si-hsa_circ_0063526 agomir treatment group and the control group. All lesions were cystic. Lesions in si-hsa_circ_0063526 agomir treatment group were relatively small. (Figure 11a)

Besides, the pathological difference of the endometrial part is further compared by the histological area. The histological area of all lesions was observed under the microscope to exclude muscle and destructed parts. The histological area of endometriosis in abdominal lesions of the miR-141-5p treatment group was significantly smaller than the two control groups ($p < 0.05$, Figure 11b-e).

3.9 Endometriosis-related gene expression differences

RT-qPCR was used to detect the gene expression differences associated with endometriosis. Compared with the control group, some genes known to promote the development of endometriosis were decreased in the si-hsa_circ_0063526 agomir group. MAPK-14, K-ras, N-cadherin, Vimentin, ER- α , ER- β were decreased in the si-hsa_circ_0063526 agomir group ($P < 0.05$ Figure 12). The decrease folds of quantitative gene expression were 5.4 times of K-ras, 3.4 times of MAPK14, 7.5 times of ER- α , 5.2 times of ER- β , and 2.1 times of N-cadherin. The expression levels of E-cadherin and Zona occludens 1 (Zona Occludens 1) in the si-hsa_circ_0063526 agomir group were increased ($P < 0.05$, Figure 12).

Immunohistochemical results showed that E-cadherin staining intensity was significantly increased in the si-hsa_circ_0063526 treatment group (Figure 13). The results are consistent with the overall research results, indicating that our research results have good stability and reliability.

Discussion

Endometriosis is a common estrogen-dependent chronic disease that affects about 10% women of childbearing age. Its histomorphology is benign, but its clinical behavior shows similar characteristics of malignant tumor' invasion, metastasis, and implantation(3).

The study of circRNA in endometriosis is still in its infancy, and its pattern and potential role in endometriosis tissues have not been elucidated. (20, 28, 29). Most of the studies on circRNA are

preliminary, and more in-depth studies are needed to provide a sufficient basis for circRNA to be a diagnostic marker or therapeutic target for endometriosis(21, 24, 25, 30).

We verified hsa_circ_0063526 was increased in endometriosis lesions, and the invasion, migration, and proliferation of End1/E6E7 cells in endometriosis patients were decreased after knockdown of hsa_circ_0063526. At the same time, bioinformatics has shown that hsa_circ_0063526 may bind miR-141-5p as a target, the dual-luciferase reporter assay further suggesting miR-141-5p can be combined with hsa_circ_0063526 and in patients' endometriosis lesions the expression of both has a negative correlation, our preliminary studies suggest that miR-141-5p regulates the development of endometriosis, It suggests that hsa_circ_0063526 may play a regulatory role in the occurrence and development of endometriosis through miR-141-5p.

Endometriosis is also known as "benign cancer". It is characterized by aggressive metastatic growth and a high risk of recurrence, similar to a malignant tumor(31, 32). So, they have something in common in terms of pathogenesis. EMT endows cells with the ability to invade and metastasize, including reducing apoptosis, suppressing the immune response, and obtaining stem cell characteristics. It not only plays a key role in embryonic development but is also involved in tissue healing, organ fibrosis, and the development of cancer(33). Simpson's theory of menstrual blood reflux suggests that EMT is one of the pathogenesis of endometriosis. Small mesothelial cells after EMT no longer provide a lamellar protective barrier between the basal layer and the lacunae. Because there is no protective barrier, endometrial cells tend to adhere to the peritoneal matrix, resulting in endometriosis. The expression of epithelial markers in endometriosis was down-regulated and the expression of mesenchymal markers was up-regulated(34). In addition to changes in cell markers, there is also evidence that EMT plays a key role in endometriosis. For example, cells die as soon as they leave ECM(extracellular matrix) or stick to the wrong place, a phenomenon known as anoikis, which is one of the challenges to Simpson's reflux hypothesis. EMT can resist apoptosis and assist the metastasis of ectopic lesions(35). Second, the endometrium is formed by the transformation of the stromal cells during the development of the urogenital system of the embryo. Due to the retention of some stromal origin imprints, endometrial epithelial cells tended to revert to the original stromal state through EMT(36, 37). We found that the EMT activity in the si-hsa_circ_0063526 group was decreased, and the expression level of E-cadherin, the epithelial marker, was increased.

The Ras/MAPK signaling pathway is important in embryo development, differentiation, proliferation, cell death, etc. K- ras is upregulated in endometriosis. K- ras activation is a classical method to establish a mouse model of spontaneous endometriosis(38, 39). Here, we found that the expression level of K-ras was lower in the miR-141-5p and si-hsa_circ_0063526 group.

Compared with linear RNA, circRNAs have a more stable structure and can resist the degradation of multiple RNA enzymes. Therefore, circRNAs could be a diagnostic and therapeutic target for endometriosis(40, 41). Currently, no studies have been reported on the role of hsa_circ_0063526 in endometriosis, but other circRNA studies have shown that circRNA has potential value in prognosis

prediction or early diagnosis of endometriosis. For the treatment of endometriosis, several possible therapeutic targets may be extracted from this regulatory pathway.

Endometriosis is considered to be an estrogen-dependent disease, ER- α and β have important roles in endometriosis. A lot of studies also used microRNA to treat estrogen-dependent disease in breast cancer. Let-7 miRNAs were down-regulated in breast cancer-initiating cells. In these cells, the recovery of miR-let-7 resulted in a decrease of proliferation in NOD/SCID mice in vitro, and decrease tumorigenesis and metastasis(42). ER- α / β expression was significantly inhibited with miR-141-5p and si-hsa_circ_0063526 suggesting that si-hsa_circ_0063526/miR-141-5p pathway blocks estrogen stimulation in the treatment. These data reveal that miR-141-5p and si-hsa_circ_0063526 therapy may specifically block the sex hormone pathway in patients with endometriosis without systemic side effects of estrogen deficiency.

In this study, miR-141-5p and si-hsa_circ_0063526 were used for local therapy. After systematic administration, oligonucleotides are difficult to reach the desired tissue because they are degraded by the liver and removed from the blood.(43). Therefore, many vectors have been used to improve the stability of oligonucleotides. However, these drugs are difficult to achieve targeted drug delivery(18). We believe that miR-141-5p and si-hsa_circ_0063526 have the best therapeutic effect as an intraperitoneal injection(44). However, since the animal model could not completely simulate the patient's condition, the model could not reflect the role of inflammation, angiogenesis, and other activities in endometriosis, and the side effects of the therapy could not be observed as thoroughly as the patient. Before being used in humans, dose-response and safety studies should be conducted.

In summary, miR-141-5p and si-hsa_circ_0063526 therapy reduce endometriosis development. The pleiotropic nature of miR-141-5p and si-hsa_circ_0063526 therapy suggests that multiple complementary mechanisms play a role in endometriosis. These effects suggest that endometriosis may be treated more comprehensively without the systemic side effects of current drugs. But further dose-response and safety studies are needed before they can be used in humans.

Abbreviations

| symbol | full name |
|---------|------------------------------------|
| EMs | endometriosis |
| N-cad | N-cadherin |
| E-cad | E- cadherin |
| EMT | Epithelial-mesenchymal transition |
| ncRNAs | non-coding RNA |
| circRNA | circular RNA |
| miRNA | microRNA |
| RNA | Ribonucleic Acid |
| ZO-1 | Zona occludens 1 |
| PVDF | polyvinylidene difluoride |
| 3 'UTR | 3 ' - untranslation region |
| ceRNA | competing endogenous RNA |
| BS | back splicing |
| RNAi | RNA interference |
| siRNA | small interference RNA |
| EdU | 5-acetylene 2' -deoxyuridine |
| PBS | phosphate buffer saline |
| VEGF | Vascular endothelial growth factor |

Declarations

Contributors

Zhangming Wei contributed the central idea, analyzed most of the data. Mengmeng Zhang participate in writing the initial draft of the paper. Lipin Li contributed to refining the ideas, carrying out additional analyses. Xiaoling Fang finalizes the revision of this paper. Xinyue Zhang has verified the underlying data.

Declaration of interests

No conflict of interest exists.

Acknowledgments

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Data sharing Statement

All data are available to others with investigator support.

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Figures

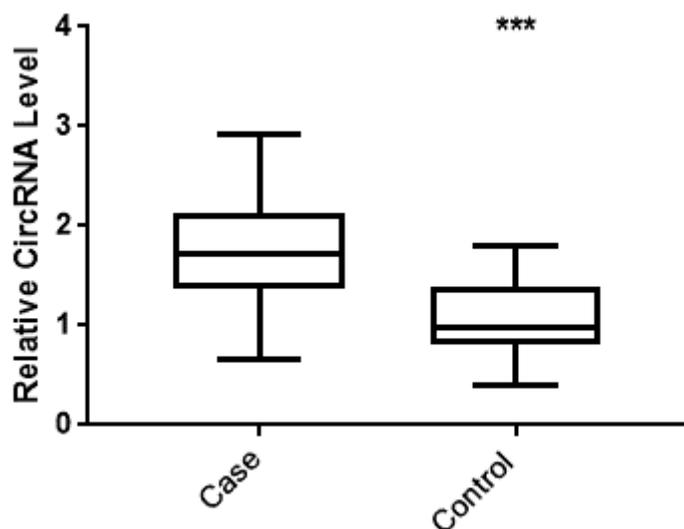


Figure 1

Quantitative analysis of the hsa_circ_0063526 level in the endometriosis group and control group by RT-qPCR. n=41 (***) P<0.005)

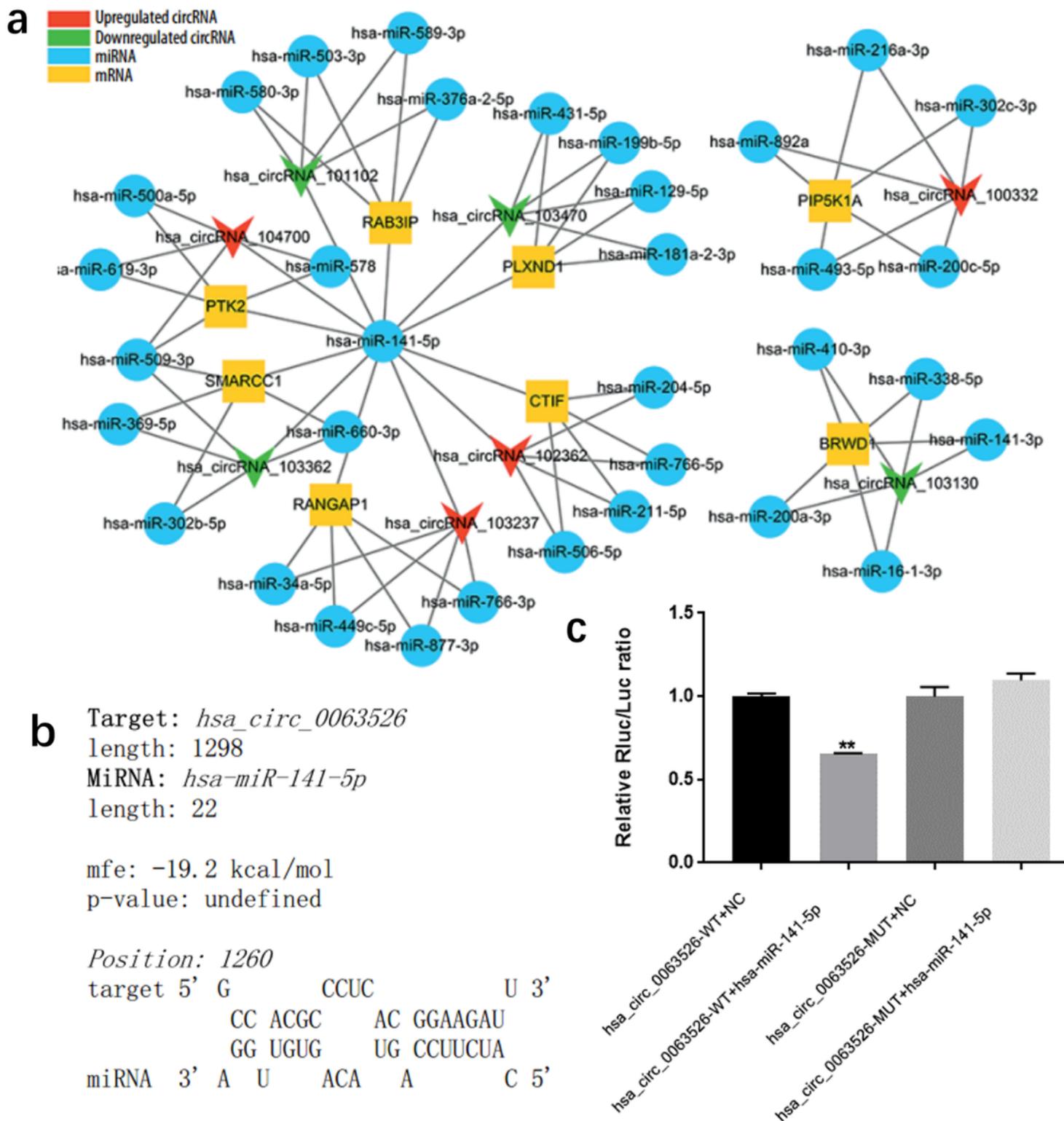


Figure 2

hsa_circ_0063526 have a binding site with miRNA-141-5p. a) circRNA/miRNA/mRNA network analysis was performed on downregulated and up-regulated circRNAs in endometriosis, and their target miRNAs and mRNAs were presented (the previous research results of our research group, *hsa_circRNA_103237* in the figure is *hsa_circ_0063526*, see reference(27)). b) *hsa_circ_0063526* complementary sequence with

miR-141-5p. c) Luciferase reporter experiment showed that miR-141-5p can bind to hsa_circ_0063526 at this site. Replicated 3 times involving 3 samples (** P<0.01).

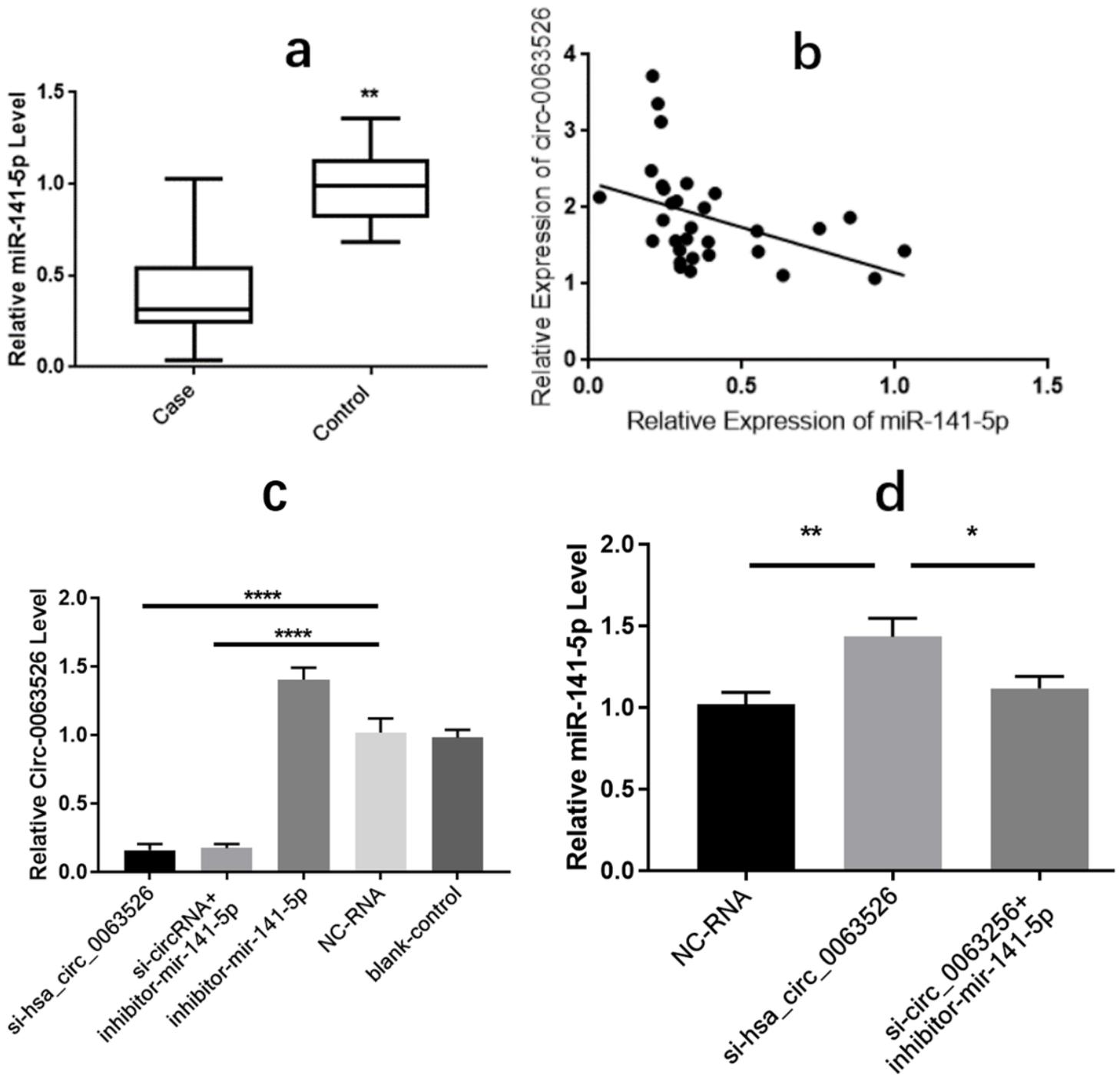


Figure 3

Hsa_circ_0063526 and miR-141-5p expression level are negatively correlated. a) Quantitative analysis of miR-141-5p expression levels in endometriosis group and control group by RT-qPCR. n=41 (** P<0.01). b) Pearson correlation analysis of the correlation between hsa_circ_0063526 and miR-141-5p expression level in the endometriosis group. n=31 (P<0.05) c) RT-qPCR shows the relative expression level of hsa_circ_0063526 in End1/E6E7 cells after downregulation of hsa_circ0063526 or inhibition of miR-141-

5p. (**** P<0.001). d) RT-qPCR shows the relative expression level of miR-141-5p in End1/E6E7 cells after down-regulation of hsa_circ_0063526. (*P<0.05, ** P<0.01). Replicated 3 times involving 3 samples

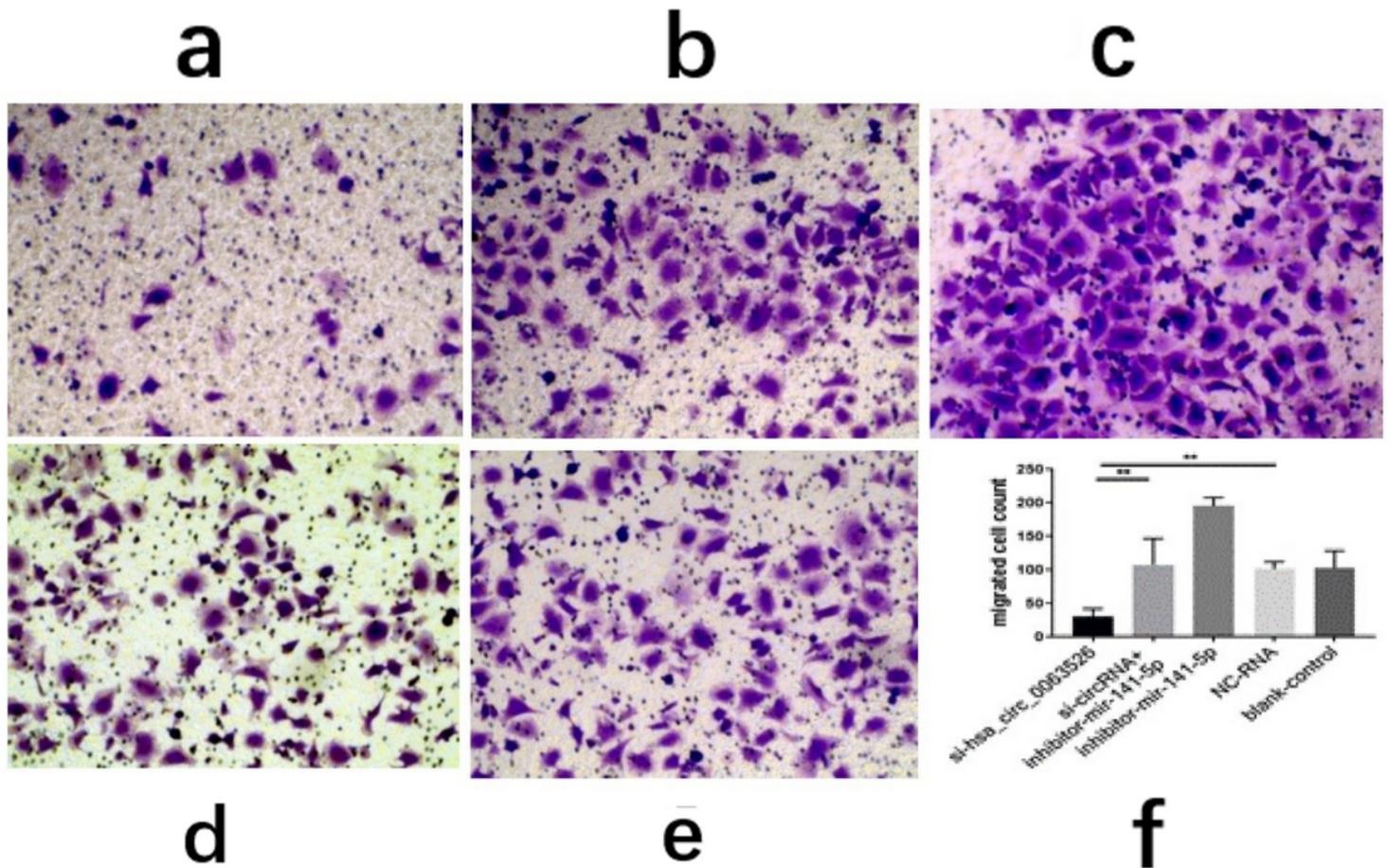


Figure 4

Transwell assay to investigate the effect of inhibition of hsa_circ_0063526 or/and miR-141-5p on cell invasion of endometriosis. (a) si-hsa_circ_0063526 group;(b) si-hsa_circ_0063526 +inhibitor miR-141-5p group;(c) miR-141-5p Inhibitor group;(d) NC-RNA group; (e) Blank control group; (f) The number of cells comparing to the NC-RNA control group. after End1 / E6E7 cell transfected hsa_circ_0063526 siRNA, the cells entering the lower chamber through the pore was lesser compared to the control group, cell invasion ability decreased. After co-transfection with si-hsa_circ_0063526 + inhibitor miR-141-5p, the invasion ability of endometriosis cells was rescued. Replicated 3 times involving 3 samples (* P<0.05, ** P<0.01).

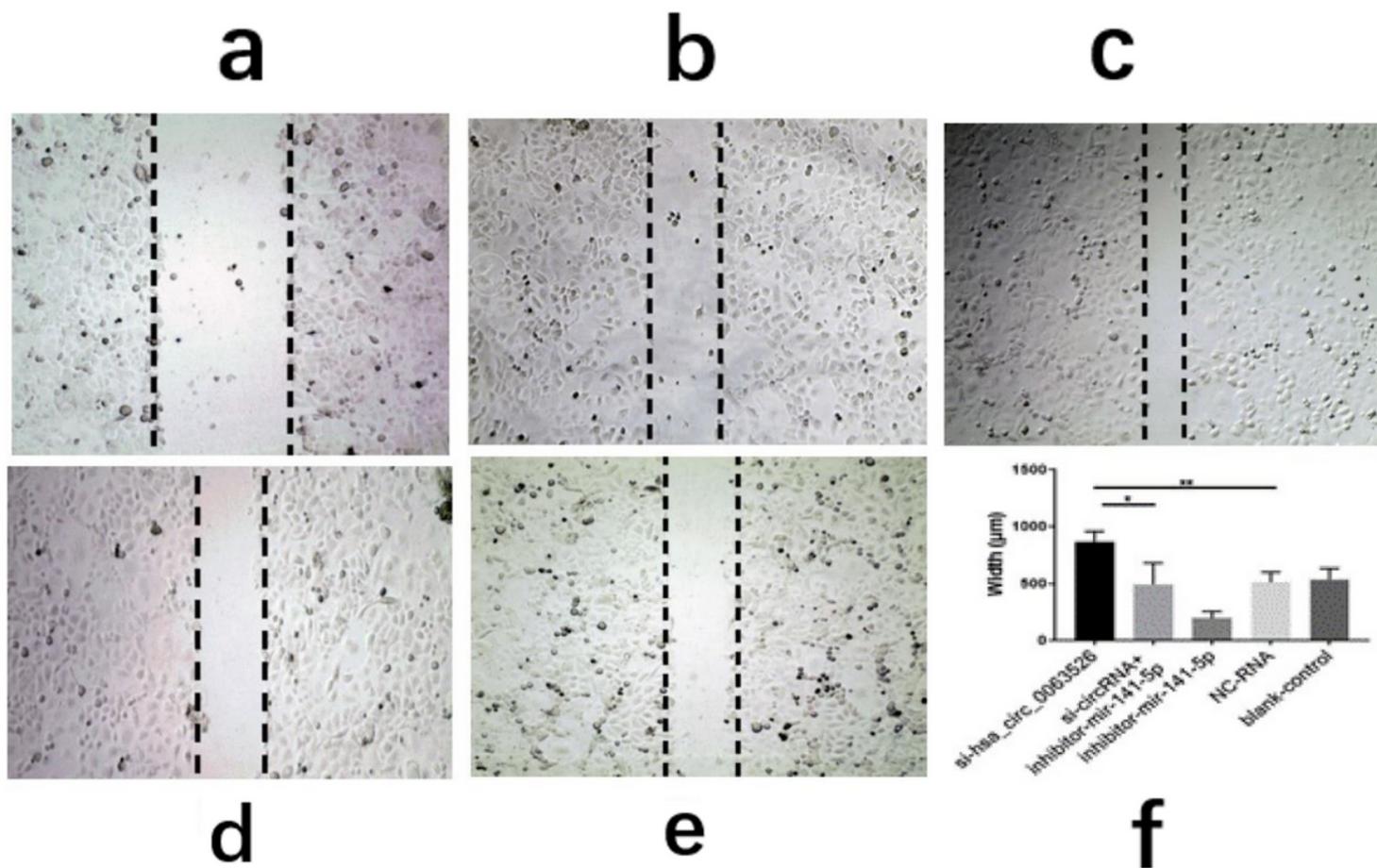


Figure 5

Wound-healing test on the effect of inhibition of hsa_circ_0063526 or/and miR-141-5p on cell migration in endometriosis. (a) si-hsa_circ_0063526 group;(b) si-hsa_circ_0063526 +inhibitor miR-141-5p group;(c) miR-141-5p Inhibitor group;(d) NC-RNA group; (e) Blank control group; (f) The scratch width comparing to the si-NC group. After transfection with hsa_circ_0063526 siRNA in End1/E6E7 cells, the cell migration ability was decreased compared with the control group. After co-transfection with si-hsa_circ_0063526 + inhibitor miR-141-5p, the cell migration ability of endometriosis was rescued. Replicated 3 times involving 3 samples (* P<0.05, ** P<0.01).

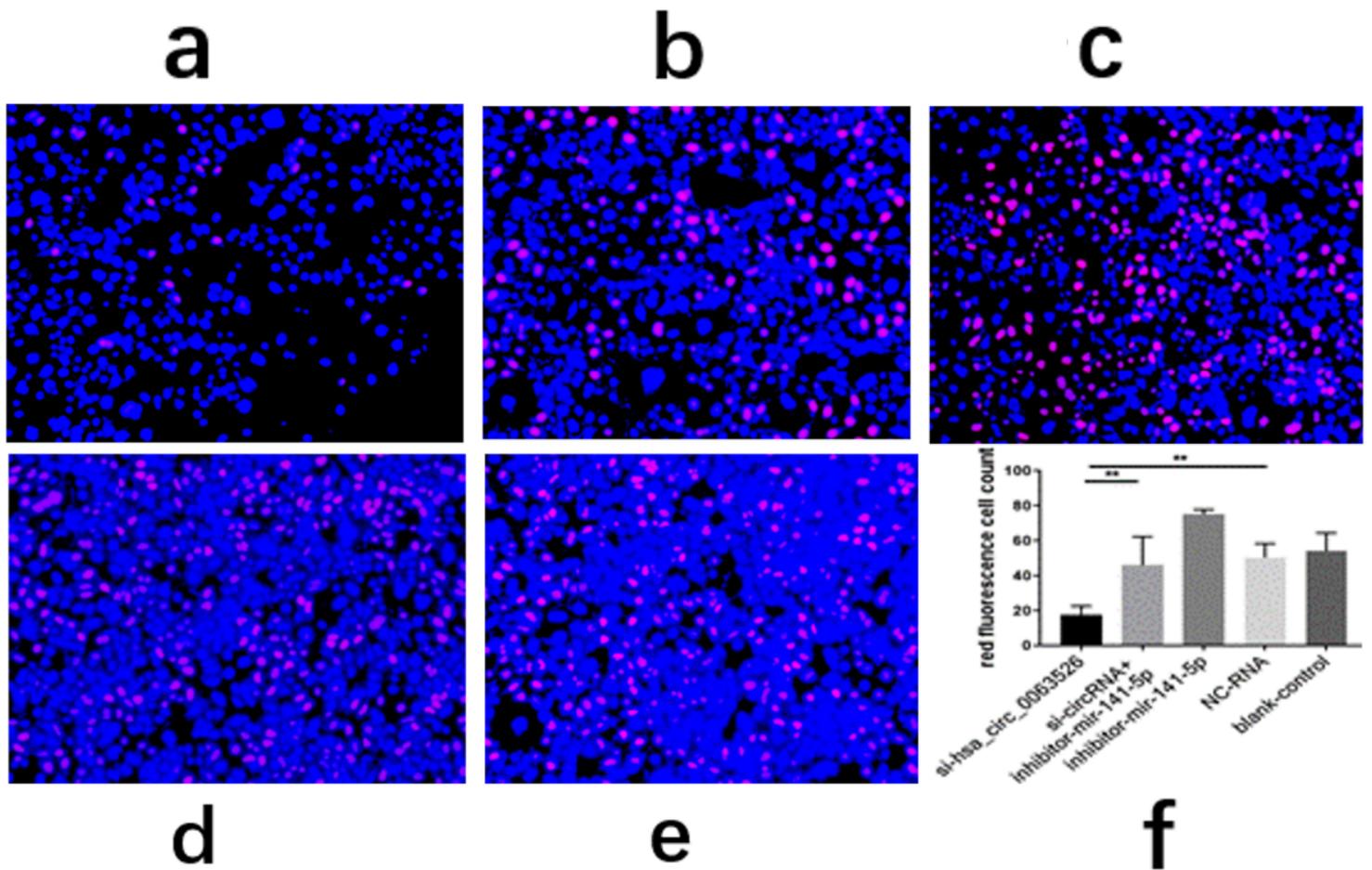


Figure 6

EdU assay was used to investigate the effect of inhibition of hsa_circ_0063526 or/and miR-141-5p on the proliferation of endometriosis cells. (a) si-hsa_circ_0063526 group;(b) si-hsa_circ_0063526 +inhibitor miR-141-5p group;(c) miR-141-5p Inhibitor group;(d) NC-RNA group; (e) Blank control group;(f) the number of red fluorescent cells. After transfection with hsa_circ_0063526 siRNA in End1/E6E7 cells, the number of cells in the control group was lower, and the proliferation ability of cells in endometriosis was decreased. After co-transfection with si-hsa_circ_0063526 + inhibitor miR-141-5p, the proliferation ability of endometriosis cells was rescued. Replicated 3 times involving 3 samples (* P<0.05, ** P<0.01).

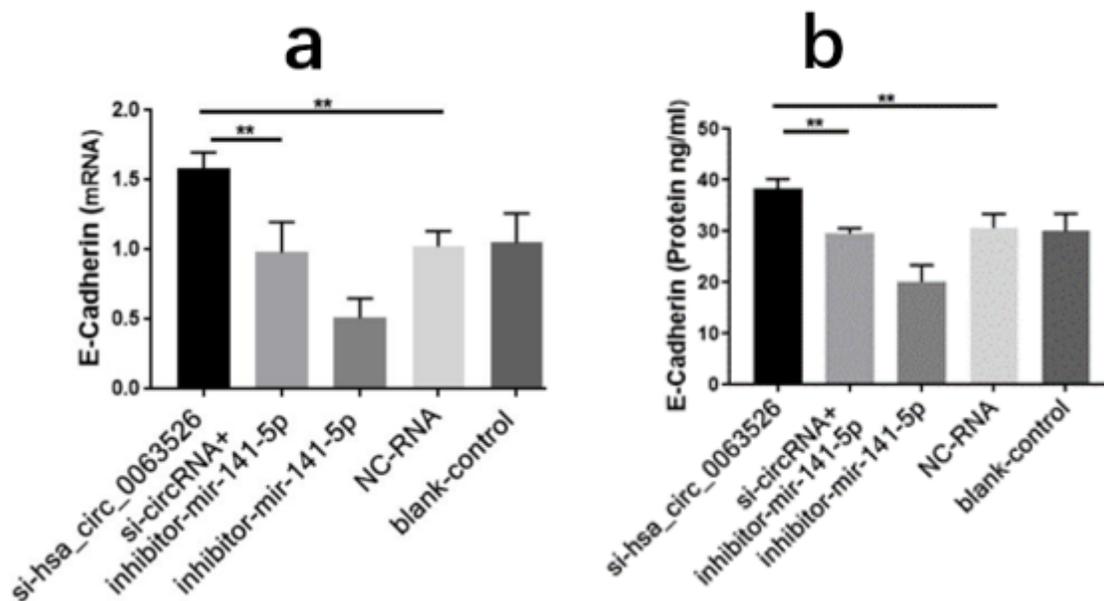


Figure 7

RT-qPCR and ELISA assay to investigate the effect of inhibition of hsa_circ_0063526 or/and miR-141-5p on the expression of E-cadherin in End1/E6E7 cell. (a) The expression level of E-cadherin mRNA (b) Expression level of E-cadherin protein. E-cadherin expression was up-regulated. Expression changes were recovered after co-transfection with si-hsa_circ_0063526 + inhibitor- miR-141-5p. Replicated 3 times involving 3 samples (* P<0.05, ** P<0.01).

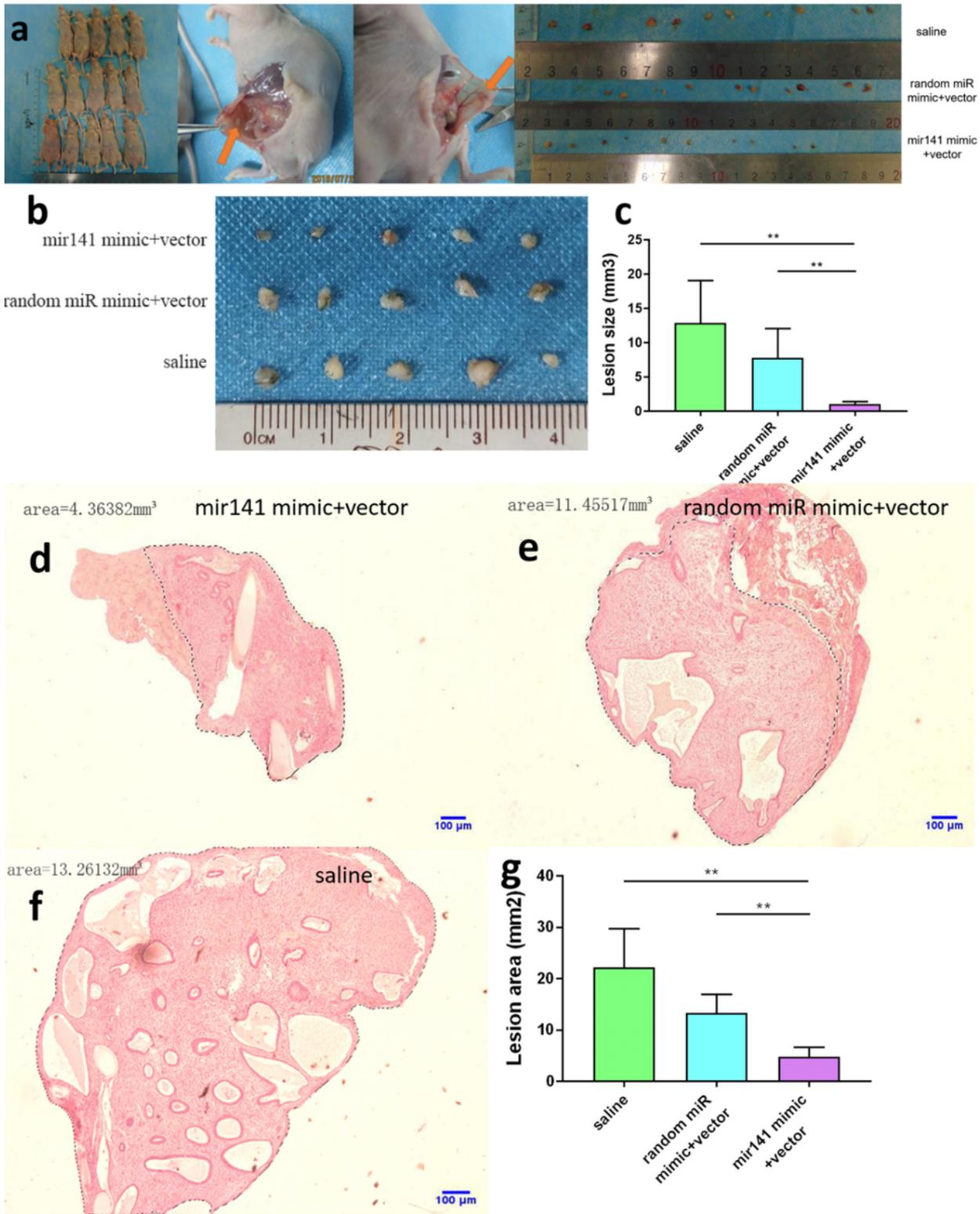


Figure 8

Collection and comparison of lesion tissues from each mouse. a): The collection of endometriosis lesions. b,c): Comparison of left abdominal lesion volume; (5 mice in each group). d-g): The difference of histological area of the median section of endometriosis in mice of the upregulated group and control group under H&E staining, *P < 0.05, ** P < 0.01.

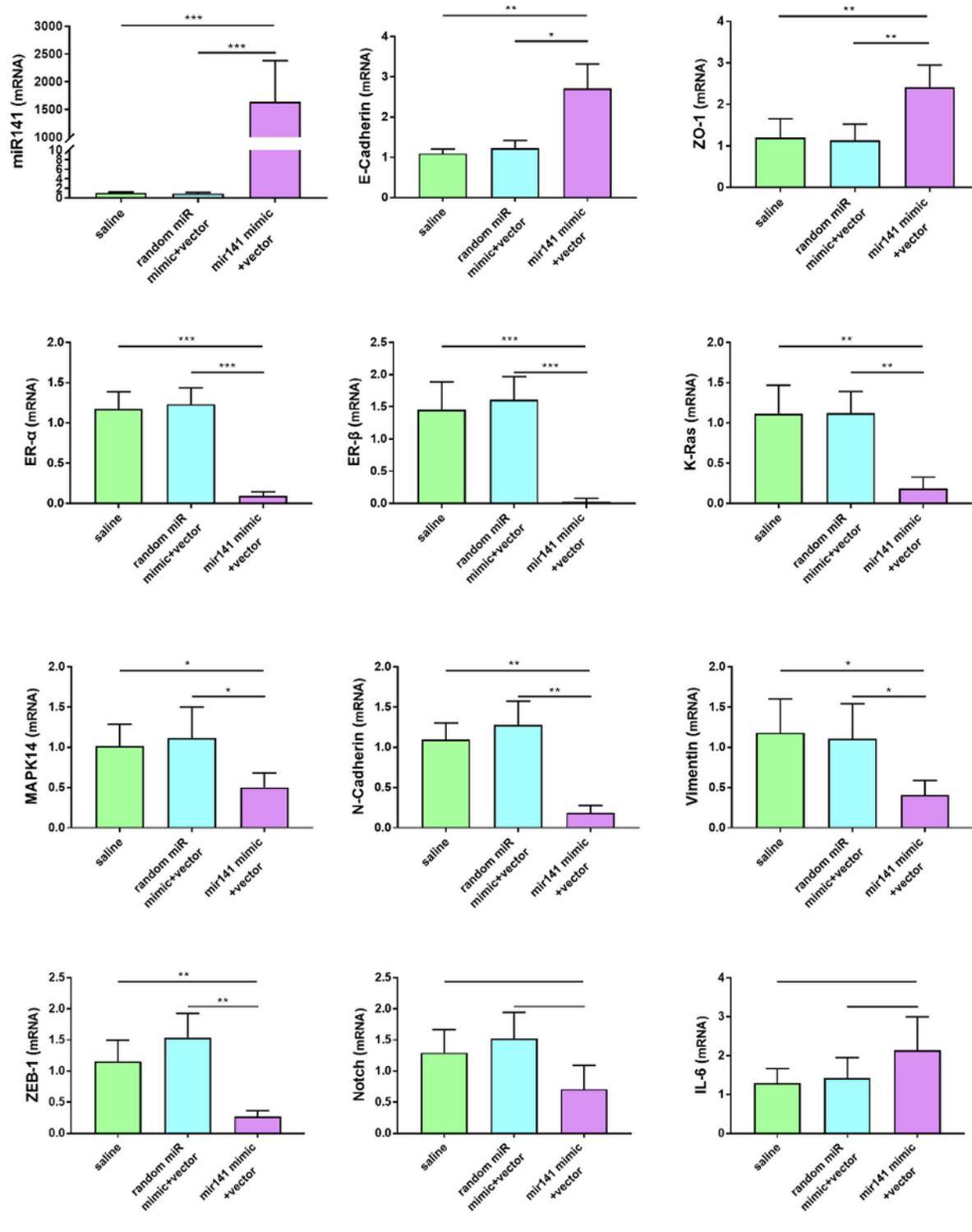


Figure 9

Effects of miR-141-5p treatment on mRNA expression of pathologically related genes in endometriosis detected by RT-qPCR. miR-141-5p treatment significantly decreased n-cadherin, Vimentin, K-ras, MAPK-14, ER-α, ER-β, ZEB-1 expression levels, and increased E-cadherin, ZO-1 expression levels. The data were shown as average percentage \pm SEM, Replicated 3 times involving 3 samples (*P < 0.05, ** P < 0.01).

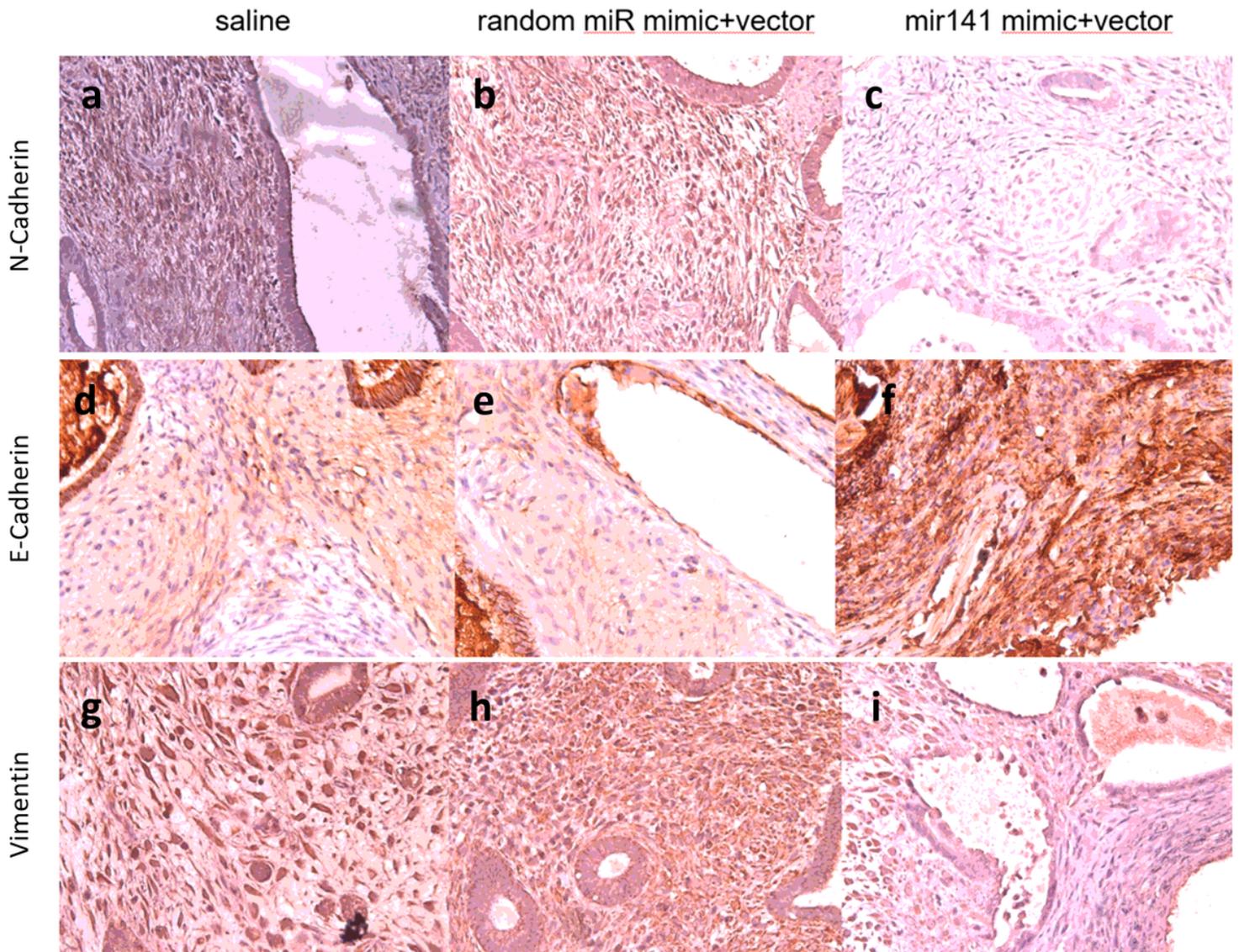


Figure 10

Representative images of N-cadherin, E-cadherin, and Vimentin protein levels detected by immunohistochemistry. N-cadherin protein (a, b, c), E-cadherin protein (d, e, f), Vimentin protein (g, h, i). Note: The lumen wall is the part of the glandular epithelium that is concerned in this study. Magnification: $\times 100$

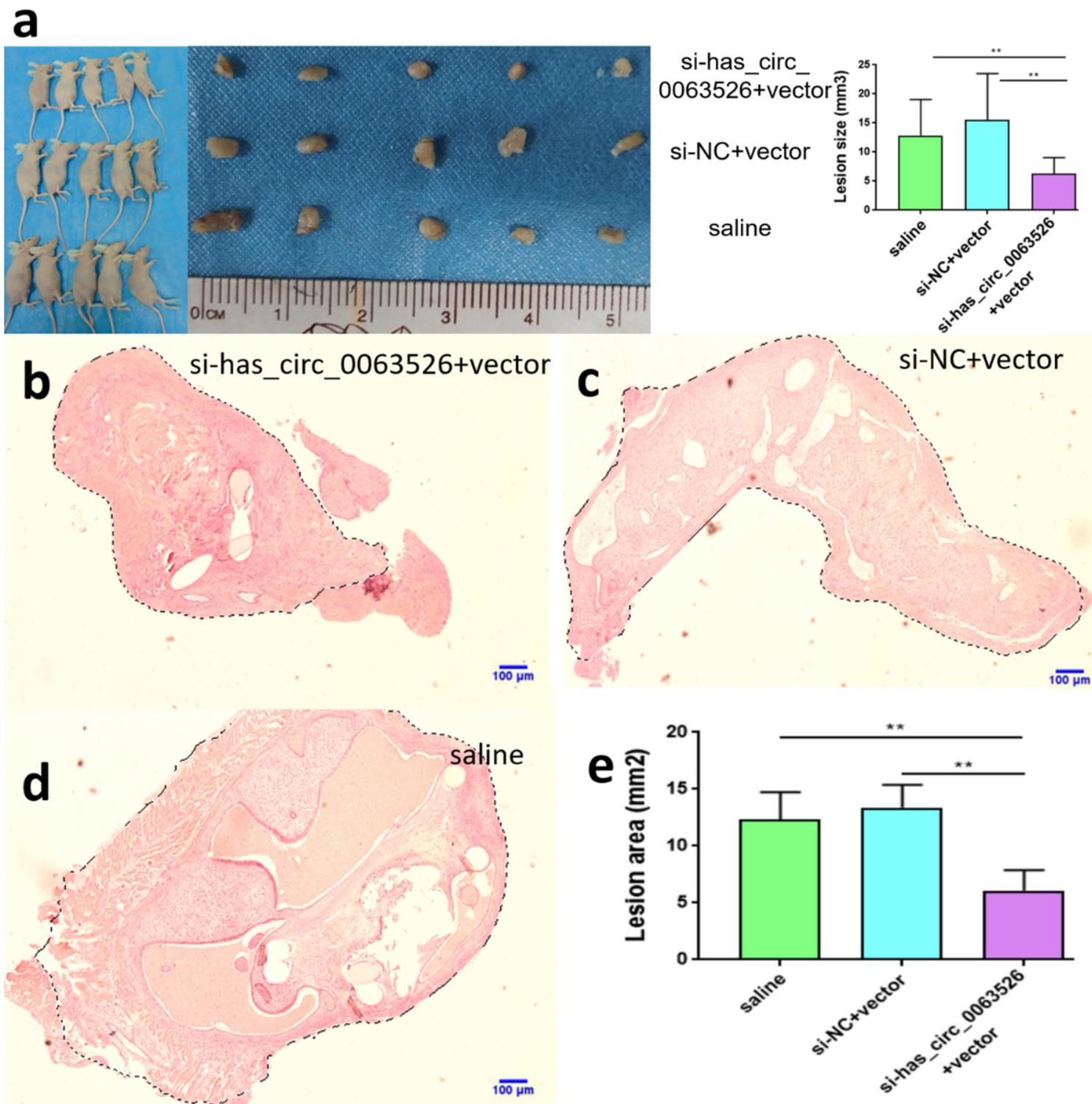


Figure 11

tissue collection and comparison of each mouse. a: Comparing the volume of lesions in the left abdomen; (5 mice in each group). b, c, d, e The difference of histological area of the median section of endometriosis in mice of the si-hsa_circ_0063526 treatment group and control group under H&E staining. Data were expressed as mean \pm SEM, * $P < 0.05$, ** $P < 0.01$.

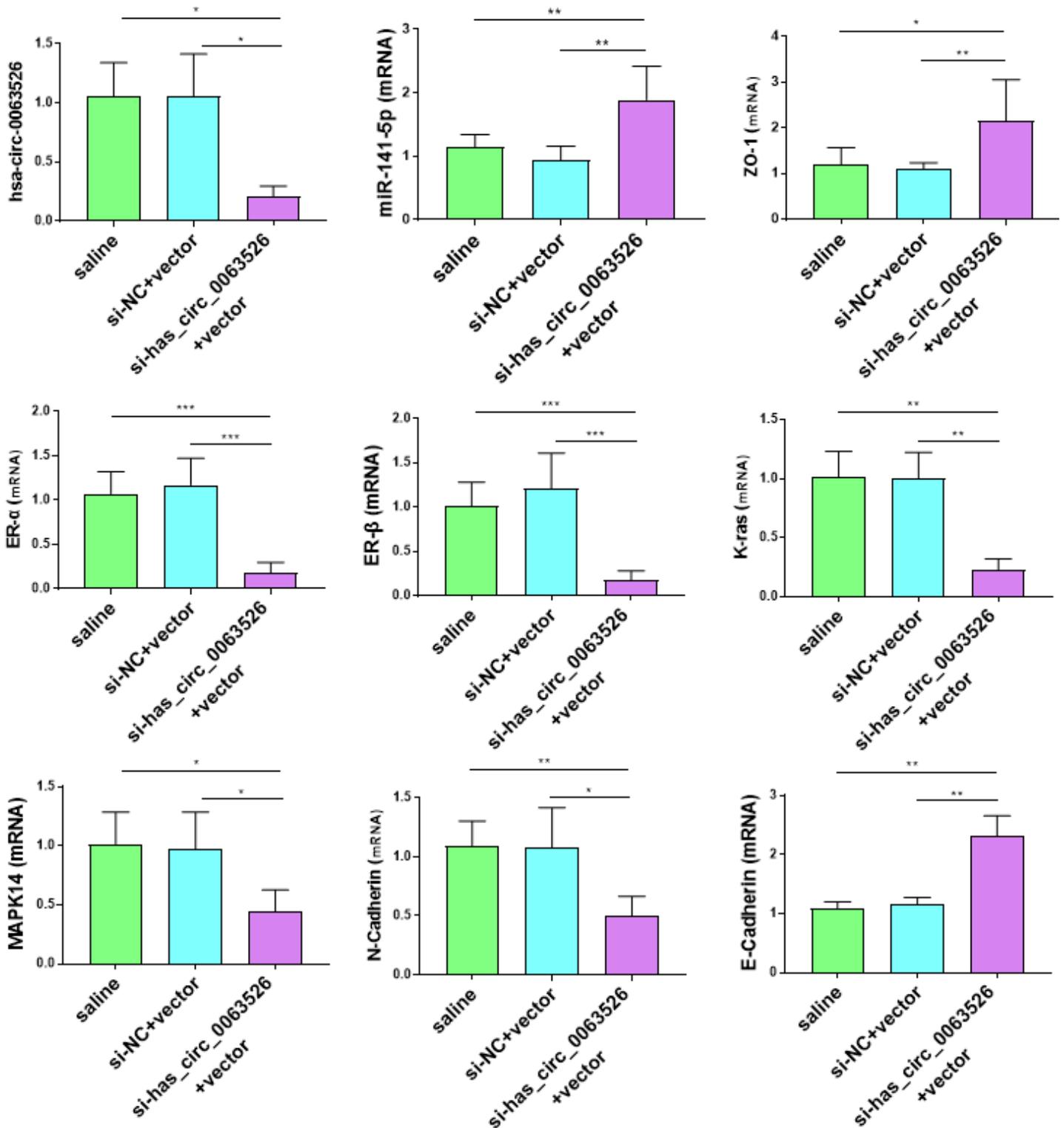


Figure 12

Effects of si-hsa_circ_0063526 treatment on mRNA expression of pathologically related genes in endometriosis detected by RT-qPCR. The expression levels of N-cadherin, K-ras, MAPK-14, ER- α , ER- β , in the si-hsa_circ_0063526 treatment group were significantly decreased, while the expression levels of E-cadherin and ZO-1 were increased. The data were shown as average percentage \pm SEM. Replicated 3 times involving 3 samples (* $P < 0.05$, ** $P < 0.01$).

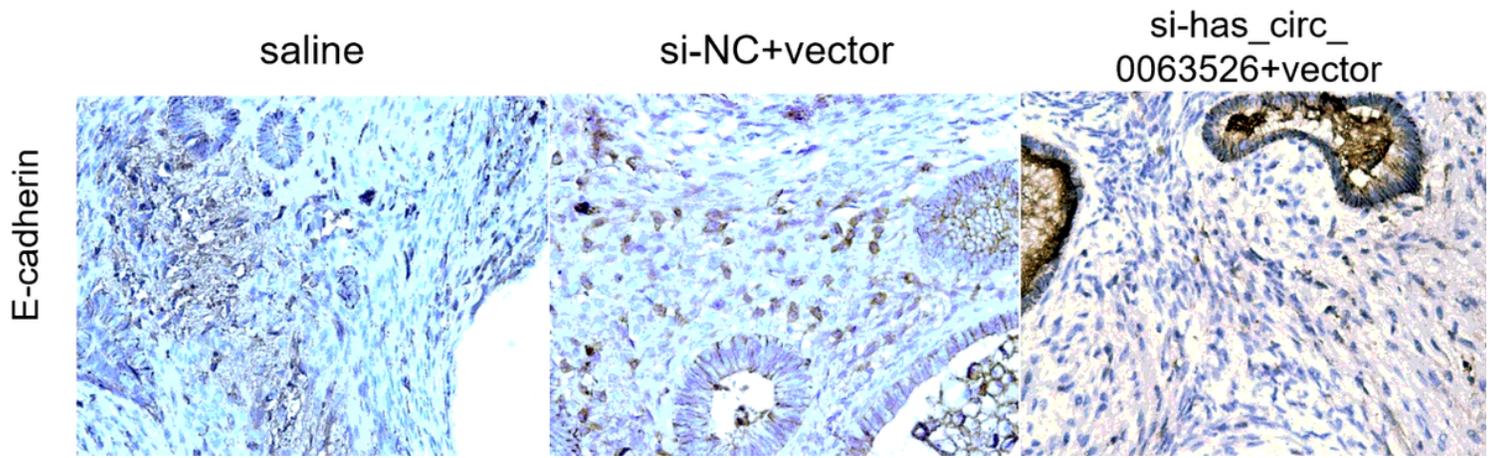


Figure 13

Representative images of E-cadherin protein levels detected by immunohistochemistry. Note: The lumen wall is the part of the glandular epithelium that is most concerned in this study. Magnification: $\times 100$.

Supplementary Files

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