Overexpression of *FLI1* promote *PART1* transcription in the endometrial epithelial cells contributing to reduced endometrial receptivity and embryo implantation failure (#85553)

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Overexpression of *FLI1* promote *PART1* transcription in the endometrial epithelial cells contributing to reduced endometrial receptivity and embryo implantation failure

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Background. Fertilization-embryo transfer in vitro (IVF-ET) is the most common and effective assisted reproductive technology for the treatment of infertility. However, recurrent implantation failure (RIF) is the biggest obstacle to the success of IVF-ET, and has become a major problem to be solved urgently in assisted reproduction. The purpose of this study is to explore the role and mechanism of FLI1 in endometrial receptivity and RIF. **Methods.** Single-cell assay for transposase accessible chromatin sequencing (scATAC-seq) was used to analyze the chromatin in luteal endometrial tissue from RIF patients and control patients. Cell experiments and in vivo ICR mice experiments were conducted to investigate the effects of FLI1 on embryo implantation ability. Chromatin immunoprecipitation (CHIP) and dual luciferase reporter assay were performed to explore the regulatory mechanism of FLI1 on PART1 transcription and expression in endometrial epithelial cells.**Results.** FLI1 was found to be specifically expressed in the endometrial epithelial cells of RIF patients. Overexpression of FLI1 in endometrial epithelial cells inhibited embryo implantation ability, while FLI1 silencing had the opposite effect. Injecting adeno-associated virus (AVV) FLI1 into the uterine horn of pregnant mice also significantly inhibited embryo implantation ability. FLI1 binding promoted PART1 transcription and expression in the PART1 promoter region in endometrial epithelial cells. Salvage experiments demonstrated that FLI1 regulated embryo implantation by promoting PART1 expression. PART1 was significantly upregulated in luteal endometrial tissue of RIF patients and cell lines of non-receptive endometrial epithelial (HEC-1-A). Overexpression of PART1 in endometrial epithelial cells significantly inhibited embryo implantation ability, while PART1 silencing had the opposite effect. Injection of AVV PART1 into the uterine horn of pregnant mice also significantly inhibited embryo implantation ability.**Conclusion.** FLI1 overexpression in RIF endometrial epithelial cells inhibited embryo implantation ability by binding to the PART1 promoter region to promote PART1 expression. These findings may

lead to the development of new targets for treating RIF.

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Abstract

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Conclusion. FLI1 overexpression in RIF endometrial epithelial cells inhibited embryo implantation

ability by binding to the PART1 promoter region to promote PART1 expression. These findings may lead to the development of new targets for treating RIF.

Introduction

In recent years, assisted reproductive technology (ART) has been increasingly used in the treatment of female and male causes of infertility. Among them, in vitro fertilization-embryo transfer (IVF-ET) is a commonly used and has enhanced the possibilities for treatmenting infertility successfully. However, the repeated implantation failure (RIF) is a key problem affecting further improvement of clinical pregnancy rates. The etiology of RIF is complex and highly heterogeneous, including endometrial receptivity, embryo aneuploidy male factors, thrombophilias, immunology, microbiome, and anatomical abnormalities [1, 2]. Among them, abnormal endometrial receptivity is one of the key factors leading to RIF, and about two-thirds of implant failures are caused by decreased endometrial receptivity [3]. In addition to the cyclical changes in endometrium morphology, increasing evidence indicated that there exists a very complex network for regulation of endometrial receptivity and implantation, including gene expression, protein modification, and miRNAs regulation [4-6]. However, it still remains unclear about the regulatory mechanism for endometrial receptivity. The critical cell fate decisions involved in this essential biological process occur at the individual cell level. Despite the apparent coordination in cellular systems, analyzing single cells has revealed that even within the same cell line or tissue, variations in genomes, transcriptomes, and epigenomes can occur during cell division and differentiation. Recent technological advancements have made it possible to measure gene-expression profiles at the level of individual cells using single-cell RNA sequencing (scRNA-seq). This breakthrough provides a unique opportunity to precisely define the cell types and molecular pathways involved in maintaining tissue homeostasis and disease [7]. Here, we firstly used scRNA-seq to make the chromatin analysis of luteal endometrial tissue to identify candidate genes, which specifically expressed in endometrial epithelial cells in patients with RIF. The mechanism of how candidate genes affect endometrial receptivity and thus play a role in RIF, has been further revealed by cell experiments and mouse animal experiments.

Materials and Methods

Patient enrollment and sample collection

This study included 20 patients with RIF who had no clinically clear etiology as the trial group. Simultaneously, 20 patients with clinical pregnancy obtained through 1-2 embryo transfers were recruited as controls, who undergo in vitro fertilization solely due to male factors. Patients of both groups were assisted with artificial pregnancy through in vitro fertiliezation/intra-cytoplasmic sperm injection-embryo transfer (IVF/ICSI-ET). In the trial group, all patients have failed clinical pregnancy, after 2-3 transplants, or with a cumulative number of high scoring blastocyst embryos transferred of 4 or more, or with a cumulative number of high scoring blastocysts transferred of 2-3. To reduce interference and ensure the credibility of the results, the

patient would be excluded from this study if combined with other diseases. Patients were also excluded who have received hormone therapy or other uterine procedures in the past 3 months. Endometrial tissue was harvested in 6 to 8 days after ovulation in the natural cycle (embryo implantation window). This research received approval from the Ethics Committee at the First Affiliated Hospital of Zhengzhou University(No.2020-KY-256). Written informed consent was obtained from all patients who participated in the study.

Mononuclear cells' preparation

Wash the endometrial tissue obtained from the patient with pre-cooled PBS buffer and immerse it in EDTA to reduce the stability of the cell membrane. Subsequently, endometrial tissue was separated into single cells using protease/collagenase. Finally, prepare mononuclear cells using single cell nucleus separation solution. The prepared mononuclear cells were qualitied for ScRNA-seq.

Library construction and RNA sequencing

Library construction and RNA sequencing were conducted according to the reported method [8]. In brief, calculate 50,000 cells, wash with PBS, add 50μl lysate after centrifugation, and centrifuge at 800×g at 4°C for 10 minutes to obtain cell nuclei. Then proceed immediately to the transposition reaction: prepare the transposition reaction Mix (including the reaction buffer, Nextera Tn5 transposase (we have optimized the amount of enzyme used), and ribozyme-free water), and use the prepared cell nucleus with the transposition reaction Mix Resuspend, mix well and incubate at 37°C for 30 minutes, and then use Qiagen purification kit to purify the DNA, and then elute the transposition reaction DNA into 10 μl of eluate. And prepare a PCR reaction system to amplify the transposable DNA (first perform a pre-qPCR to evaluate the number of PCR cycles), then purify the amplified product, use Bioanalyzer for quality control and Kapa Library Quantification Kit to quantify the library (observe whether There is a typical nucleosome distribution pattern), you can go to the computer for high-throughput sequencing.

Generation of single-cell gene expression matrices

Each nucleus of the qualified sample is mixed with reagents and magnetic beads with barcode sequence successively on the 8-channel microfluidic chip, and then wrapped by oil drops to form Gel Beads in extrusion (GEMs) containing single nuclei and single gel beads. Each gel bead carries several sequences that are combined by Illumina connector sequence (P5), 16bp 10x cell barcode (Barcode), and some Illumina read1 primers.

After the library construction is completed, Qubit 2.0 is used for preliminary quantification, followed by Agilent Bioanalyzer High Sensitivity DNA Chip to detect the Insert DNA of the library. After the Insert size meets expectations, qPCR method is used to accurately quantify the effective concentration (2 nM) of the library to ensure the quality of the library. After passing the library inspection, perform Nextseq 500 platform sequencing.

Quality control, cell-type clustering, and major cell-type identification

To ensure high-quality data, perform offline data quality control measures, such as verifying sequencing quantity, barcode carrying rate, and sequencing quality Q30 value. Additionally, conduct Q30 quality control on barcode and sample index sequences to ensure high resolution at both the cell and sample levels.

The nucleosome, which is composed of DNA and histone, is the basic structural unit of a chromosome. Each histone octamer consists of two histone molecules, with about 200 bp of DNA coiled around the octamer's core structure to form a nucleosome. Within these 200 bp, 146 bp coil directly around the histone octamer's core and are resistant to nuclease digestion, while the remaining DNA links the next nucleosome. Nucleosome localization refers to the precise determination of nucleosome location on the genome, which is always accompanied by gene transformation from inhibition to transcription. Nucleosome localization has significant research value in transcriptional regulation, DNA replication and repair, and is currently a hot topic in epigenetic research. Based on nucleosome structural characteristics and the distribution of ATAC-seq inserted fragments, fragments within 147 bp of inserted fragments are designated as nucleosome deletion regions, while fragments between 147-294 bp are considered nucleosome distribution regions, providing accurate nucleosome localization.

The size distribution of ATAC-seq insertion fragments provides information on the packaging and location of nucleosomes, while the fragment length captures the periodicity of nucleosome positioning. Fragments less than 147 bp indicate nucleosome-free regions, whereas fragments of approximately 147-294 bp suggest a single nucleosome distribution region. The relationship between the length and quantity of insertion fragments also exhibits periodic fluctuations due to the nucleosome structure, with fluctuation frequency correlated with the length or multiple of unit nucleosome.

To evaluate the number of cells captured in a barcode-labeled multi-cell mixed sample sequencing library, peak calling and insertion fragment data alignment to peak regions are performed. The number of cells successfully captured is determined by evaluating the insertion fragment count and length covering the peaks region. Based on barcode and corresponding insertion fragment count statistics, cells are classified into cellular and non-cellular categories. Cells that exceed the threshold of captured insertion sequences are classified as single cells, while those with insufficient captured insertion sequences are classified as non-cells.

10x Genomics allows for the simultaneous sequencing of libraries from multiple cells to obtain ATAC data for multiple cells. The Cell Ranger ATAC package uses the Python library MOODS (https://github.com/jhkorhonen/MOODS) to scan for peak matches with group positions in each cell and annotate peaks using the JASPAR database to group cells. Two-dimensional cell clustering results are obtained using the tSNE dimensionality reduction algorithm.

Detection of FLI1 expression level in primary endometrial epithelial cells

Transfer freshly collected endometrial tissue from the implantation stage to a culture dish and cut into a paste. Digest with collagenase and incubate on a shaker at 37 ℃ for 30 minutes. Filter with a filter to remove endometrial stromal cells and obtain primary endometrial epithelial cells. After inoculating the obtained cells into a culture dish for 24 hours, extract total RNA and total protein.

FLI1 expression levels were assessed using real-time quantitative PCR (RT-qPCR) and Western blot (WB) analysis.

Source and Analysis of lncRNA Data

The datasets GSE26787 and GSE111974 were downloaded from the GEO database, including gene expression data of 5 and 24 pairs of endometrial tissue from pregnant women and endometrial tissue from patients with RIF, respectively. Based on the similarity of lncrna expression levels, a clustering heatmap was drawn using the heatmap package in R software, with $|logFC| > 1.5$ and p-value < 0.01 as the criteria. Thus, differential expression of lncRNAs between RIF and control groups was obtained. Subsequently, the intersection of two sets of different lncRNAs is used to obtain the key lncRNAs that regulate the RIF process.

Target relationship verification of FLI1 and PART1

The targeting relationship of *FLI1* and *PART1* was revealed through the Chromatin Immunoprecipitation (ChIP) assay in endometrial epithelial cells. In brief, crosslinking and cracking are firstly performed to stabilize the complex and separate the nuclear components with anti-FLI1 or anti-anti-IgG. Then DNA fragmentation is realized through chromatin fragmentation, and protein DNA complexes were captured and separated by immunoprecipitation. Subsequently, cross-linking and DNA purification were performed. Finally, determine the enrichment differences of *PART1* among different groups. The report of double Luciferase was used to further verify the target relationship between *FLI1* and identified lncRNA (*PART1*).

Cell lines and cell culture

Both the non receptive endometrial epithelial cell line (HEC-1-A) and the receptive endometrial epithelial cell line (Ishikawa, RL95-2) were purchased from Wuhan Punosai Life Technology Co., Ltd (Hubei, China). The above cell lines have stable phenotypes and can effectively simulate in vitro experiments of primary endometrial epithelial cells. BeWo cells are sourced from Wuhan Punosai Life Technology Co., Ltd (Hubei, China). They were cultured in dulbecco's modified eagle medium (DMEM) medium and at 37 ℃, saturated humidity, and 5% CO_2 incubator. By subculture the cell line, adjust the cell state, and take logarithmic phase cells for subsequent cell experiments.

Construction of FLI1 overexpression / silencing vectors and Obtaining stable transfected cell lines

The *FLI1* overexpression plasmid (OE-FLI1) and the corresponding negative quality control plasmid (OE-NC) were constructed by Shanghai Hanheng Biotechnology Co., Ltd (Shanghai, China). The *FLI1* silencing plasmid (shFLI1) and the corresponding negative quality control plasmid (shNC) were constructed by Guangzhou Huijun Biotechnology Co., Ltd (Guangdong, China).

The sequences of shFLI1 and shNC are as follows:

CCGGCGTCATGTTCTGGTTTGAGATCTCGAGATCTCAAACCAGAACATGACGTTTTT GAATT (shFLI1#1);
CCGGCCCATGAACTACAACAGCTATCTCGAGATAGCTGTTGTAGTTCATGGGTTTTT GAATT (shFLI1#2);
CCGGCCCTTCTGACATCTCCTACATCTCGAGATGTAGGAGATGTCAGAAGGGTTTTT GAATT (shFLI1#3);
CCGGTCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTT TGAATT (shNC).

Ishikawa and HEC-1-A in the logarithmic phase were randomly divided into four groups: OE-NC group, OE-FLI1 group, shNC group, and shFLI1 group. The above two types of cells were transfected with overexpressed empty vector (OE-NC), overexpressed FLI1 vector (OE-FLI1), silenced control vector (shNC), and silenced FLI1 vector (shFLI1), respectively. The transfection reagent uses Lipo8000™ (Beyotime Biotechnology, Shanghai, China). After 24 hours of transfection, the cell line stably expressing the aforementioned vector was obtained.

Cell function assays

Detection of the effect of *FLI1* on the proliferation ability of endometrial epithelial cells using CCK-8 kit (Beyotime Biotechnology, Shanghai, China) according to the instructions. The effect of *FLI1* on the migration of endometrial epithelial cell migration was evaluated by scratch test. Image J (National Institutes of Health, Bethesda, USA) was used to calculate the area value of scratches. The migration rate was calculated as: (0 h Area-24 h Area)×100% / 0 h Area. Evaluation of the effect of *FLI1* on the epithelial mesenchymal transition of endometrial epithelial cells by detecting the protein expression levels of Vimentin and E-cadherin using WB assay. Evaluating the Effect of *FLI1* on Embryo Adhesion Using the BeWo Cell Ball Adhesion Model.

Mouse model construction and adenovirus infection

We procured sexually mature male and female mice of Specific Pathogen Free (SPF) grade from Skbex Biotechnology Co., LTD., (Heinan, China) with the males being fertile. All of them were ICR strain and 8-week old, and they were caged for mating, according to 1:2 ratio of male: female. Pregnant female mice were confirmed through vaginal suppositories and selected for subsequent in vivo experiments.

Epithelial cell specific overexpression of *PART1* overexpression adeno-associated virus (AAV-PART1) and its corresponding negative control adeno-associated virus (AAV-NC) were constructed and packaged by Shanghai Hanheng Biotechnology Co., Ltd.(Shanghai, China), with a virus titer of 1.00×10^{12} VG/mL. AAV-NC and AAV-PART1 were injected into the mouse uterine cavity through the uterine horn, respectively. On days 0, 2, 5, and 8 of injection, endometrial epithelial cells were isolated, and the overexpression efficiency of *PART1* was detected by RT-qPCR and WB.

Corneal injection experiment

12 pregnant mice were randomly divided into two groups: AAV-NC group and AAV-PART1 group, with 6 mice in each group. The Ethics Committee at Zhengzhou University approval for this research (No.ZZU-LAC20200828[08]). All mice were housed in SPF animal room with temperature of 24-26 ℃ and humidity of 50-60%. They were fed irradiated feed appropriate for their needs and provided with sterile water. The animal room operated on a 12-hour light/dark cycle, with lights on from 6:00 AM to 6:00 PM. To provide environmental enrichment, each cage contained nesting material and chew sticks. When the pregnant mice were 1.5 days pregnant, they were anesthetized with isoflurane inhalation and placed prone on a small animal operating table insulated at 37 ℃. The mouse's back was prepared with skin, and the skin was fully exposed. A small incision on the back was performed to fully expose the mouse's uterine horn. Subsequently, use an insulin syringe to aspirate 20 μ L-adeno-associated virus was injected into the uterine horn (AAV-NC group mice were given AAV-NC, and AAV-PART1 group mice were given AAV-PART1).After injection into the uterine horn, suture the wound, and when the mouse regains consciousness, send it back to the animal room for separate feeding. On the 9th day of pregnancy in mice, blue dye was injected into the tail vein to euthanize the mice, then all mice were euthanized using a CO2 euthanasia chamber. The chamber was initially filled with a mixed gas environment of 6:4 CO2 and O2, and the CO2 concentration was gradually increased to 100% after the animals lost consciousness. The mice were kept in the chamber for a minimum of 10 minutes to confirm death, during which they were completely unconscious. There were no surviving animals in the end of study. The uterus was removed to observe the number of embryo implantation.

Statistical analysis

All data in this study were calculated using mean ± standard deviation (SD). Graphpad Prism 6.0 was used for data statistical analysis and plotting. Student's t-test or Fisher 's exact test was used to analyze the differences between groups. P value < 0.05 is considered statistically significant.

Results

Single cell clustering analysis and cell group identification in endometrial tissue

Cluster analysis and cell gene labeling were performed on the data obtained from single cell sequencing to identify 8 types of cell populations, including stromal fibroblast cells, endometrial cells, epithelial cells, T cells, B cells, dendritic cells (DC cells), smooth muscle cells and neural progenitor cells in endometrial tissue (Figure 1A). TSNE nonlinear dimensionality reduction analysis was performed on the cell populations in the RIF and control groups, and the visualized results are exhibited in Figure 1B. Furthermore, heat maps were used to display the top 10 differentially expressed genes in each cell group (Figure 1C). Thus, the key genes in each cell group were analyzed, which can serve as biomarkers for identifying cell types. The results showed that *CDH11* was a biomarker for stromal fibroblasts, *SPINK4* for endometrial cells, DMD for epithelial cells, CD247 for T cells, CD70 for B cells, CD83 for dendritic cells, ITGA8 for smooth muscle cells, and UBE2C for neural progenitor cells (Figure 1D-K).

RIF patients possessing a low proportion of endometrial epithelial cells and high FLI expression

Statistical analysis was conducted on the frequency and proportion of different cell populations in the RIF and NC groups. Compared with the NC group, the proportion of interstitial fibroblasts and epithelial cells significantly decreased in the RIF group, while the proportion of endothelial cells, T cells, and B cells significantly increased. Among them, the difference in epithelial cells between the RIF group and the NC group was the most significant (0.17% *vs* 20.94%, Figure 2A). The above results reveal that endometrial epithelial cells are closely related to low endometrial receptivity in RIF patients and may be participate in RIF.

Assay for transposase accessible chromatin with high-throughput sequencing (ATAC-seq) analysis revealed that chromatin accessibility was significantly different between RIF and control endometrium. Most of the different peaks lie in the enhancer position (defined as enhancer when the distance from TSS is>2kb). At the same time, we conducted ChiP seq experiments on the active enhancer histone modified marker H3K27Ac, and found that most of these up-regulated peaks are enriched with H3K27Ac, suggesting that these peaks are mainly located in the active enhancer region, which may be related to gene transcription activation. Transcription factor (TF) motif analysis (MEME software) was performed on these peaks, and it was found that the most significantly enriched motif was *FLI1*, which was 65% enriched among all differentially upregulated peaks. Figure 2B showed the peak distribution of FLI1. Further analysis showed that chromatin accessibility of FLI1 in endometrial epithelial cells of RIF group was significantly higher than that of control group (logFC=0.27, p = 0.009). This findings suggests that FLI1 has a significant regulatory effect on embryo implantation.

RT-qPCR and WB were used to detect the expression levels of *FLI1* in primary epithelial cells obtained from endometrial tissues of the RIF and control groups. The findings revealed a substantial increase in the mRNA and protein expression of FLI1 in endometrial epithelial cells of the RIF group compared to control patients (Figure 2C-D). The above data indicate that the accessibility of transcription factor FLI1 chromatin in endometrial epithelial cells of RIF patients is enhanced, and the expression level of *FLI1* is also significantly up-regulated, which reveals that *FLI1* is involved in the process of RIF's low endometrial receptivity.

In vitro study on the effect of FLI1 on cell function

Non receptive endometrial epithelial cell lines (HEC-1-A) and receptive endometrial epithelial cell lines (both Ishikawa and RL95-2) were selected for assays in vitro. According to the RT-qPCR results, the expression level of FLI1 was significantly higher in HEC-1-A cells compared to Ishikawa and RL95-2 cells (3.91±0.50 *vs* 0.81±0.15 *vs* 1.02±0.12, $p < 0.0001$, Figure 3A). That is to say, *FLI1* is highly expressed in non receptive endometrial epithelial cells. Stable transfection lines were establishment by overexpression and silencing of FLI1 plasmid transfection into Ishikawa cells and HEC-1-A cells (Figure 3B-C). Silencing *FLI1* can significantly increase the proliferation ability of HEC-1-A cells ($p < 0.0001$). On the contrary, overexpression of *FLI1* can significantly inhibit the proliferation ability of Ishikawa cells (p <

0.0001, Figure 3D). The above data indicate that overexpression of *FLI1* inhibits endometrial epithelial cell proliferation, while silencing *FLI1* promotes endometrial epithelial cell proliferation. Scratch test showed that silencing *FLI1* significantly increased the migration ability of HEC-1-A cells (Figure 3E). Nevertheless, overexpression of FLI1 can significantly inhibit the migration ability of Ishikawa cells (Figure 3F). It indicate that overexpression of *FLI1* inhibits endometrial epithelial cell migration, while silencing *FLI1* promotes endometrial epithelial cell migration. In HEC-1-A cells, silencing *FLI1* increases the expression of Vimentin protein and inhibits the expression of E-Cadherin protein (Figure 3G-H); In Ishikawa cells, overexpression of *FLI1* significantly inhibits Vimentin protein expression and promotes E-cadherin protein expression (Figure 3G-H). It is revealed that overexpression of *FLI1* inhibits epithelial mesenchymal transition of endometrial epithelial cells, while silencing *FLI1* promotes epithelial mesenchymal transition of endometrial epithelial cells. Ishikawa cells overexpressing *FLI1* significantly weakened their ability to adhere to BeWo cell spheres (Figure 3I), while HEC-1-A cells silencing *FLI1* significantly increased their ability to adhere to BeWo cell spheres (Figure 3I). It is suggested that overexpression of FLI1 inhibits BeWo cell ball adhesion, while silencing FLI1 increases BeWo cell ball adhesion.

In vivo study on the effect of FLI1 on mouse embryo implantation

To determine the effect of *FLI1* on mouse embryo implantation, AAV-OE-NC and AAV-OE-FLI1 were injected into the uterus of female mice through the uterine horn, respectively. RT-qPCR and Western blotting were used to detect changes in FLI1 expression levels in endometrial epithelial cells of each group of mice at 0, 2, 5, and 8 days after injection. The RT-qPCR results showed a significant increase in FLI1 mRNA expression levels in female mouse endometrial epithelial cells starting from the second day of AAV-OE-FLI1 intervention (Figure 4A); Consistent with the RT-qPCR results, protein blotting also showed that the expression level of FLI1 protein in female mouse endometrial epithelial cells was significantly increased starting from the second day of AAV-OE-FLI1 intervention (Figure 4B); It is suggested that injecting AAV-OE-FLI1 into the uterine horn can overexpress FLI1 in mouse endometrial epithelial cells. Furthermore, AAV-OE-NC and AAV-OE-FLI1 were injected into the uterus of pregnant mice through the uterine horn, and it was found that the number of embryo implantation in AAV-OE-FLI1 treated mice was significantly lower than that in AAV-OE-NC treated mice (Figure 4C). The above data indicate that epithelial cell specific overexpression of *FLI1* can inhibit mouse embryo implantation.

Molecular mechanism of FLI1 inhibiting embryo implantation

In the GSE26787 dataset, a total of 22 differentially expressed lncRNAs (DElncRNAs) were identified in the endometrial tissue of pregnant women compared to those of RIF patients, of which 13 were significantly upregulated and 9 were significantly downregulated (Figure 5A). In the GSE111974 dataset, 72 DElncRNAs were identified, of which 55 were significantly upregulated and 17 were significantly downregulated (Figure 5B). Only *LOC100505912* and *PART1* were shared in DElncRNAs of GSE26787 and GSE111974, and both were significantly

upregulated in endometrial tissue of RIF patients (Figure 5C). Therefore, we speculate that the abnormal expression of LOC100505912 and PART1 in the patient's endometrial tissue may be the key lncRNAs regulating RIF.

The RT qPCR results showed that overexpression of *FLI1* in endometrial epithelial cells significantly increased the expression level of *PART1*, while silencing *FLI1* in endometrial epithelial cells had the opposite effect (Figure 6A), indicating that *FLI1* regulates *PART1* expression. However, overexpression/silencing of *FLI1* in endometrial epithelial cells did not affect the expression level of *LOC100505912* (Figure 6B). Meanwhile, using the JASPAR online (https://jaspar.genereg.net/), we found that FLI1 only had binding sites in the PART1 promoter region with a score of 0.86 (Figure 6C), while there was no binding site in the LOC100505912 promoter.

ChIP and double Luciferase reporting experiments were adopted to verify the targeting relationship between FLI1 and PART1, . The ChIP results showed that the enrichment level of PART1 in the anti FLI1 group was significantly higher than that in the anti IgG group (Figure 6D), indicating the interaction between FLI1 and PART1 promoters. The double Luciferase reporting experiment further proved that FLI1 binded to the PART1 promoter to promote PART1 transcription (Figure 6E). The above data indicated that FLI1 directly binds to the PART1 promoter to promote PART1 transcriptional expression.

In order to determine the effect of PART1 on mouse embryo implantation, AAV-OE-NC and AAV-OE PART1 were injected into the uterus of pregnant mice through the uterine horn, respectively. On the 8th day after injection, RT-qPCR was used to detect the changes in PART1 expression levels in the endometrial epithelial cells of each group of mice. The RT-qPCR results showed that intervention with AAV-OE PART1 significantly increased the expression level of PART1 mRNA in female mouse endometrial epithelial cells (Figure 7A), indicating that injecting AAV-OE PART1 into the uterine horn can overexpress PART1 in mouse endometrial epithelial cells. Furthermore, AAV-OE-NC and AAV-OE-FLI1 were injected into the uterus of pregnant mice through the uterine horn, and it was found that the number of embryo implantation in AAV-OE-PART1 treated mice was significantly lower than that in AAV-OE-NC treated mice (Figure 7A). Based on the above results, we concluded that epithelial cell specific overexpression of PART1 can inhibit mouse embryo implantation.

Further cell function experiments found that overexpression of FLI1 significantly inhibited the proliferation ability of Ishikawa cells, while silencing PART1 significantly reversed the above phenomenon (Figure 7B). The scratch test results revealed that overexpression of *FLI1* can significantly inhibit the migration ability of Ishikawa cells, while silencing *PART1* can significantly reverse the above phenomenon (Figure 7C). The results of Western blotting showed that overexpression of FLI1 reduced the expression of Vimentin protein and increased the expression of E-cadherin protein in Ishikawa cells, while silencing PART1 significantly reversed the above phenomenon (Figure 7D). Overexpression of FLI1 inhibits epithelial mesenchymal transition of endometrial epithelial cells by upregulating PART1 expression. The ability of Ishikawa cells overexpressing FLI1 to adhere to BeWo cell spheres was significantly reduced,

while silencing PART1 could significantly reverse the above phenomenon (Figure 7E).
The above results indicate that *FLI1* binding promotes the transcription and expression of *PART1* on the promoter of *PART1*. Epithelial cell specific overexpression of *PART1* inhibits mouse embryo implantation and inhibits a series of functions of endometrial epithelial cells, such as cell proliferation, cell invasion, epithelial mesenchymal transition, and BeWo cell ball adhesion. Thereby, it contributes to reduced endometrial receptivity and embryo implantation failure.

Discussion

The reasons for RIF are diverse, such as embryonic genome defects, endometrial problems, maternal autoimmune factors, sperm quality, etc. Among them, endometrial problems are one of the common causes of RIF. The endometrium is an important site for embryo implantation and development, so the structural and functional abnormalities of the endometrium directly affect the success rate of implantation. Previous study has revealed. Previous studies have revealed some of the reasons why endometrial problems affect RIF. Endometriosis can lead to abnormal proliferation and differentiation of endometrial cells, forming ectopic foci and polyps, thereby hindering embryo implantation [9]. In addition, endometriosis may also lead to uterine wall stiffness, narrowing of the lumen, and further affect implantation. Excessive thickness or thickness of the endometrium may also lead to RIF. Excessive thickness of the endometrium can affect communication between the embryo and the mother, thereby affecting embryo implantation; An excessively thin endometrium is not conducive to embryo implantation and growth [10]. Endometrial infection and inflammation can lead to abnormal local immune function in the endometrium, thereby affecting embryo implantation [11]. An abnormal endometrial microbiota has been associated with implantation failure [12]. Moreover, endometrial receptivity refers to the ability of the endometrium to allow the localization, adhesion, penetration, and implantation of blastocysts, as well as to enable embryo implantation and development [13]. Endometrial receptivity is closely related to embryo implantation and is one of the key factors determining the success of assisted reproductive technology [14]. At present, there are very limited methods for treating endometrial receptivity abnormalities, mainly due to the unclear molecular mechanism of RIF caused by endometrial receptivity. Our study revealed this molecular mechanism through the use of scATAC-seq technology combined with in vitro and in vivo studies.
Firstly, scATAC-seq and patients study results revealed RIF patients possessed a low proportion of endometrial epithelial cells and high *FLI* expression in endometrial epithelial tissue (Figure 2). Single-cell sequencing has the ability to uncover the gene structure and expression status of individual cells, providing valuable insights into structural and copy number variations, RNA expression levels, and more. This technology enables precise differentiation of cell types and facilitates the study of molecular mechanisms at the cellular level. A previous study used single-cell RNA sequencing (scRNA-seq) to identify dramatic differential expression of endometrial receptivity-related genes in four major endometrial fibroblast-like cells from patients with recurrent implantation failure (RIF), compared to the control endometrium [15]. The proportion

of CD49a+CXCR4+NK cells was observed to be reduced in individuals with RIF. Our study firstly use scATAC-seq and identified a significantly low proportion of endometrial epithelial cells in the endometrial tissue of RIF patients (Figure 2A). The peaks of FLI1 were also analyzed using scATAC-seq data, indicating that FLI1 has a significant regulatory effect on embryo implantation (Figure 2B). Endometrial epithelial cells play a critical role in embryo implantation and mid-term pregnancy, as they are one of the main components of the endometrium [16,17]. Following ovulation, these cells secrete increased amounts of mucus to provide a favorable environment for embryo transfer. Furthermore, endometrial epithelial cells secrete essential nutrients like glucose and amino acids, which aid embryonic development. Additionally, they regulate the immune response in the endometrium by secreting various bioactive molecules, such as cytokines and chemokines, preventing the immune system from rejecting the embryo. Moreover, by secreting growth factors, adhesion molecules, and other compounds, endometrial epithelial cells facilitate embryo implantation and ensure successful attachment. Beside, progesterone targets these cells, enabling them to synthesize and secrete hormones like progesterone that support pregnancy and fetal growth and development. The Ets family of transcription factors is essential for controlling cell proliferation, apoptosis, differentiation, and migration [18]. Among the members of this family, Friend leukemia integration 1 (FLI1) has gained considerable attention due to its crucial role in various physiological processes and diseases. *FLI1* is a significant regulator of normal hematopoiesis, vasculogenesis, immune response, and pro-fibrotic potential [19]. However, its role on endometrial receptivity and RIF has never been revealed. Here, *FLI1* is found to be highly expressed in non receptive endometrial epithelial cells, and overexpression of *FLI1* inhibits cell proliferation, cell migration, epithelial stromal transformation of endometrial epithelial, as well as ball adhesion of BeWo cell (Figure 3). While these abilities are crucial for embryo transfer and endometrial receptivity. For example, epithelial cells transform into mesenchymal (EMT) can mediate the growth of trofoblast, which is essential for normal function of endometrium and implantation and development of an embryo [20]. Evidence suggests that the cell adhesion molecules play a unique role in human embryo implantation [21]. Furthermore, in vivo experiments directly proved epithelial cell specific overexpression of *FLI1* can inhibit mouse embryo implantation (Figure 4). Therefore, the conclusion is drawn that epithelial cells play an important role in the process of endometrial receptivity of RIF, and *FLI1* is specifically expressed in the endometrial epithelial cells of patients with RIF, which is involved in the process of endometrial receptivity of RIF by inhibiting serious of cell ability.

LncRNAs are non-coding RNA that participate in the occurrence and development of many diseases, including RIF [22-24]. To further reveal the molecular mechanisms by which *FLI1* affects RIF and endometrial receptivity, it was preliminarily identified that lncRNAs of *PART1* and *LINC00092* may involved in the process by analysis GEO data (Figure 5). Previous studies have shown that transcription factors regulate lncRNA transcription expression by binding to the lncRNA promoter region, thereby affecting the biological function of cells [25, 26]. Therefore, we suspected that *FLI1* may regulate *PART1* and *LINC00092* transcription expression by binding

to the lncRNA promoter region. Nevertheless, overexpression/silencing of *FLI1* in endometrial epithelial cells did not affect the expression level of *LOC100505912*. We utilized the JASPAR online tool to predict *FLI1* binding sites in the promoter region of *PART1*. To validate this, we conducted ChIP and double Luciferase reporting experiments, which provided evidence that *FLI1* directly binds to the *PART1* promoter and facilitates its transcriptional expression (as shown in Figure 6). *PART1* is a crucial factor in cancer and osteogenic differentiation of bone marrow mesenchymal stem cells, as it regulates cell proliferation, apoptosis, invasion, and metastasis through various potential mechanisms [27, 28]. However, there are currently no reports of *PART1* participating in RIF and endometrial receptivity. Our study revealed that *PART1* inhibited mouse embryo implantation, as well as a series of functions of endometrial epithelial cells, such as cell proliferation, cell invasion, epithelial mesenchymal transition, and BeWo cell ball adhesion. These data suggested the molecular mechanism that *FLI1* mediated *PART1* expression inhibits embryo implantation and reduces endometrial receptivity in RIF patients. These findings would provide *FLI1* and *PART1* were potential targets for the treatment of RIF. However, this study only focused on the role of FLI1 and PART1 in endometrial receptivity and RIF and did not explore other potential factors involved in these processes. Additionally, the study was conducted in mice and cell lines, and further validation in human samples is needed to confirm the clinical relevance of the findings.

Acknowledgments

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Availability of data and material

Data Availability

The raw data can be found online at https://doi.org/10.6084/m9.figshare.22688389.v2.

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network and identification of hub genes in recurrent implantation failure. Reprod Biol Endocrinol. 2021 Jul 9;19(1):108.
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Figure 1

Single cell clustering analysis and identification in endometrial tissue.

(A) Single cell t-SNE clustering and identification in endometrial tissue; (B) Single cell t-SNE clustering and identification in endometrial tissue of the RIF and NC groups; (C) The heat map visualized the relative expression levels of the top 10 genes in each cell group; Visualization display of key genes in each cell group (D-K).

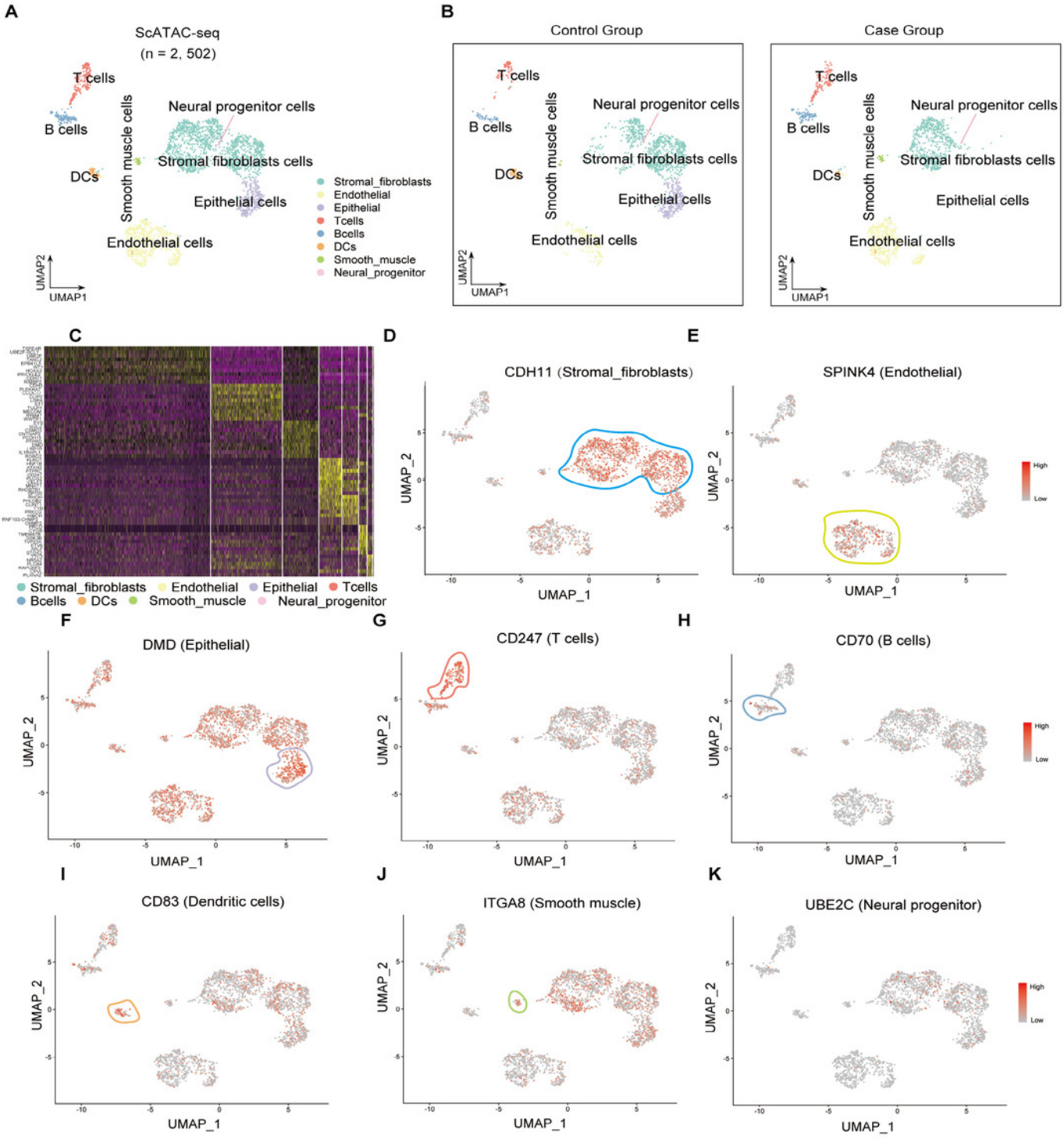

A
ScATAC-seq
(n = 2, 502)
T cells
B cells
DCs
Smooth muscle cells
Neural progenitor cells
Stromal fibroblasts cells
Epithelial cells
Endothelial cells
Stromal_fibroblasts
Endothelial
Epithelial
Tcells
Bcells
DCs
Smooth_muscle
Neural_progenitor
UMAP2
UMAP1
B
Control Group
Case Group
C
Stromal_fibroblasts Endothelial Epithelial Tcells
Bcells DCs Smooth_muscle Neural_progenitor
D
CDH11 (Stromal_fibroblasts)
E
SPINK4 (Endothelial)
High
Low
UMAP_1
UMAP_2
F
DMD (Epithelial)
G
CD247 (T cells)
H
CD70 (B cells)
I
CD83 (Dendritic cells)
J
ITGA8 (Smooth muscle)
K
UBE2C (Neural progenitor)

Figure 2

Low endometrial receptivity in RIF patients is associated with low endometrial epithelial cell content and abnormal FLI expression.

(A) The number and proportion of 8 cell populations in endometrial tissue of RIF and control groups; (B) The data of scATAC seq were used to analyze the peaks of FLI1, and the height of each color peak represents the opening degree of corresponding cell type chromatin; (C) The mRNA expression of FLI1 in endometrial epithelial cell by RT-qPCR; (D) The protein expression of FLI1 in endometrial epithelial cell by western bolt.scATAC, single cell assay for transposase accessible chromatin with high-throughput sequencing; NC, control group; #, $p < 0.05$; ##, $0.05 < P < 0.001$; ###, $0.01 < P < 0.0001$.

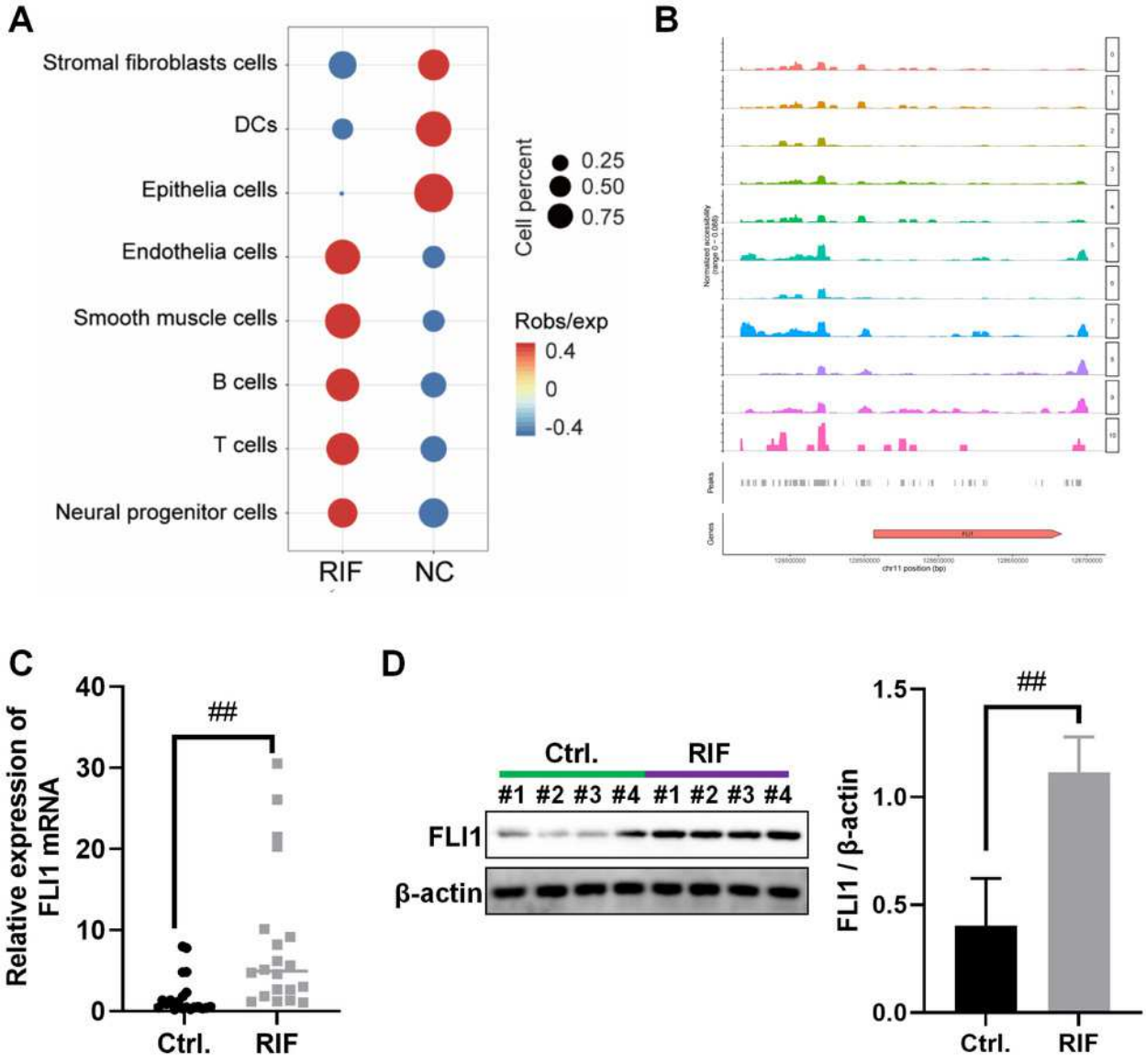

A
Stromal fibroblasts cells
DCs
Epithelia cells
Endothelia cells
Smooth muscle cells
B cells
T cells
Neural progenitor cells
RIF
NC
Cell percent
0.25
0.50
0.75
Robs/exp
0.4
0
-0.4
B
C
Relative expression of FLI1 mRNA
40
30
20
10
0
##
Ctrl.
RIF
D
Ctrl.
RIF
#1 #2 #3 #4 #1 #2 #3 #4
FLI1
β-actin
FLI1 / β-actin
1.5
1.0
0.5
0.0
##
Ctrl.
RIF

Figure 3

Cell assays revel the effect of FLI1 on cell functions.

(A) Differences in FLI1 expression levels between HEC-1-A, Ishikawa and RL95-2 by RT-qPCR; Verification of FLI1 overexpression and silencing plasmid stable transfection through RT-qPCRt (B) and Western blot (C); (D) The proliferation ability of HEC-1-A and Ishikawa cells transfected with OE-NC and OE-FLI1 plasmids with CCK-8 method; Invasive ability of HEC-1-A (E) and Ishikawa (F) transfected with OE-NC and OE-FLI1 plasmids using scratch test; Protein expression of Vimentin and E-Cadherin in HEC-1-A and Ishikawa cells transfected with OE-NC and OE-FLI1 plasmids by western blot (G-H); (I) The effect of overexpression of FLI1 in Ishikawa cells on the adhesion ability of BeWo cell spheres and the silencing of FLI1 in HEC-1-A cells on the adhesion ability of BeWo cell spheres. OE-FLI1 and OE-NC: FLI1 overexpression plasmids and their negative control plasmids; ShFLI1 and shNC: FLI1 silencing plasmid and its negative control plasmid; NC, control group; #, $p < 0.05$; ##, $0.05 < P < 0.001$; ###, $0.01 < P < 0.0001$.

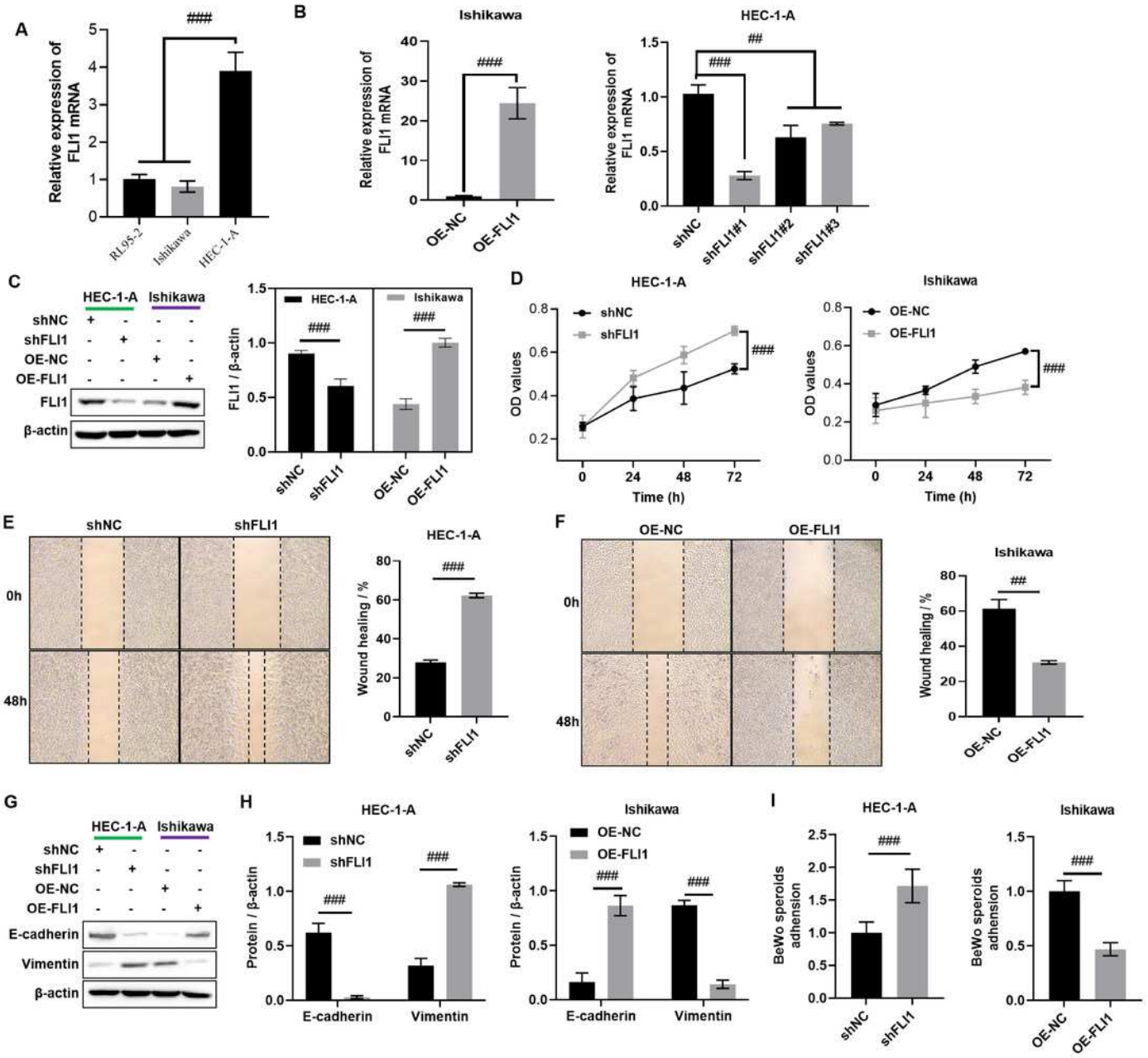

A
Relative expression of FLI1 mRNA
###
RL95-2
Ishikawa
HEC-1-A
B
Ishikawa
Relative expression of FLI1 mRNA
###
OE-NC
OE-FLI1
HEC-1-A
##
###
Relative expression of FLI1 mRNA
shNC
shFLI1#1
shFLI1#2
shFLI1#3
C
HEC-1-A
Ishikawa
shNC
shFLI1
OE-NC
OE-FLI1
FLI1
β-actin
FLI1 / β-actin
###
D
HEC-1-A
shNC
shFLI1
OD values
Time (h)
###
Ishikawa
OE-NC
OE-FLI1
E
shNC
shFLI1
0h
48h
HEC-1-A
Wound healing / %
###
F
OE-NC
OE-FLI1
Ishikawa
##
G
E-cadherin
Vimentin
H
Protein / β-actin
I
BeWo speroids adhension

Figure 4

Epithelial cell specific overexpression of FLI1 inhibits mouse embryo implantation.

The protein expression of FLI1 in mouse endometrial epithelial cells was detected by time-lapse fluorescence quantitative PCR and Western blotting (A and B); (C) The effect of epithelial cell specific overexpression of FLI1 on the number of embryo implantation in pregnant mice. AVV-OE-FLI1 and AVV-OE-NC: FLI1 overexpress adeno-associated viruses and their negative control adeno-associated viruses. # #, P<0.01; ###, P<0.001.

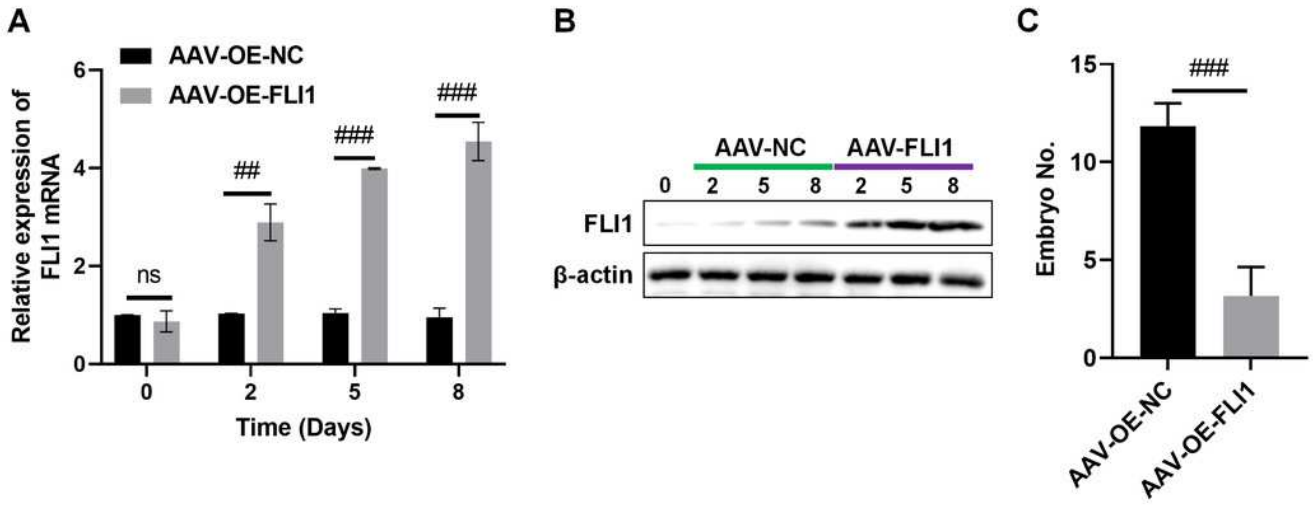


Figure 5

LOC100505912 and *PART1* are abnormally upregulated in the patient's endometrial tissue.

(A) The heat map displays the differential expressed lncRNAs in GSE26787; (B) The heat map displays the differential expressed lncRNAs in GSE111974; (C) The Venn plot and bar plot display the differentially expressed lncRNAs shared by GSE26787 and GSE111974. Ctrl and RIF: Endometrial tissue of normal pregnancy patients and endometrial tissue of patients with repeated implantation failure; DElncRNAs: differentially expressed lncRNAs## $P<0.01$.

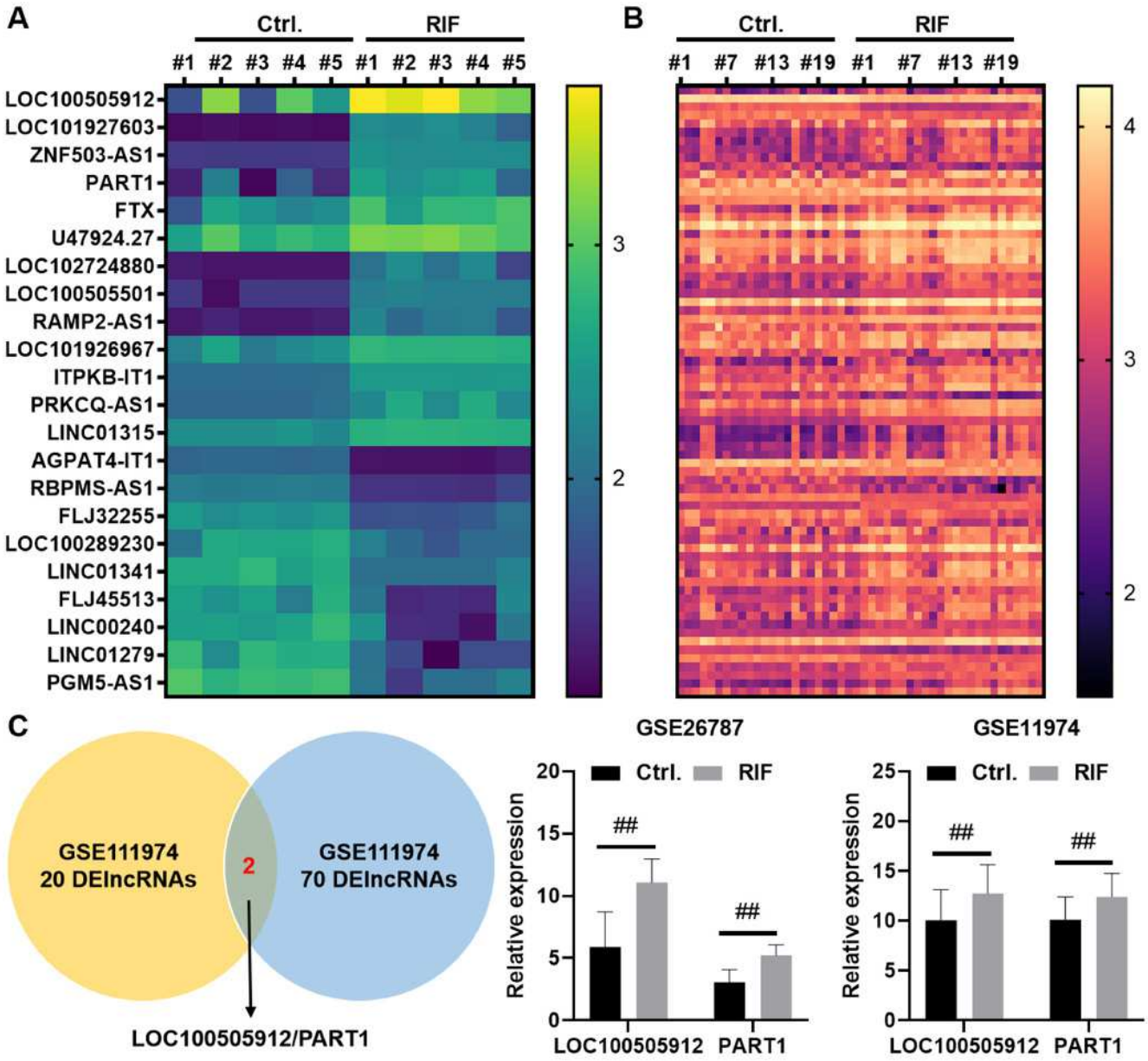

A
Ctrl.
RIF
#1 #2 #3 #4 #5 #1 #2 #3 #4 #5
LOC100505912
LOC101927603
ZNF503-AS1
PART1
FTX
U47924.27
LOC102724880
LOC100505501
RAMP2-AS1
LOC101926967
ITPKB-IT1
PRKCQ-AS1
LINC01315
AGPAT4-IT1
RBPMS-AS1
FLJ32255
LOC100289230
LINC01341
FLJ45513
LINC00240
LINC01279
PGM5-AS1
3
2
B
Ctrl.
RIF
#1 #7 #13 #19 #1 #7 #13 #19
4
3
2
C
GSE111974
20 DElncRNAs
2
GSE111974
70 DElncRNAs
LOC100505912/PART1
GSE26787
Ctrl.
RIF
Relative expression
20
15
10
5
0
##
##
LOC100505912
PART1
GSE11974
Ctrl.
RIF
Relative expression
25
20
15
10
5
0
##
##
LOC100505912
PART1

Figure 6

FLI1 binding promotes transcriptional expression of *PART1* on the promoter of *PART1*.

RT-qPCR detection of the effect of overexpression/silencing of FLI1 on *PART1* and *LOC100505912*in endometrial epithelial cells (A-B); (C) JASPAR predicted the binding site of *FLI1* on the *PART1* promoter; (D) ChIP was used to detect the interaction between FLI1 and *PART1* promoters; (E) The dual Luciferase report demonstrated that *FLI1* binds to *PART1* promoter to regulate *PART1* transcription. ShFLI1 and shNC: *FLI1* silencing plasmid and its negative control plasmid; OE-FLI1 and OE-NC: *FLI1* overexpression plasmids and their negative control plasmids## P<0.01□###P<0.001.

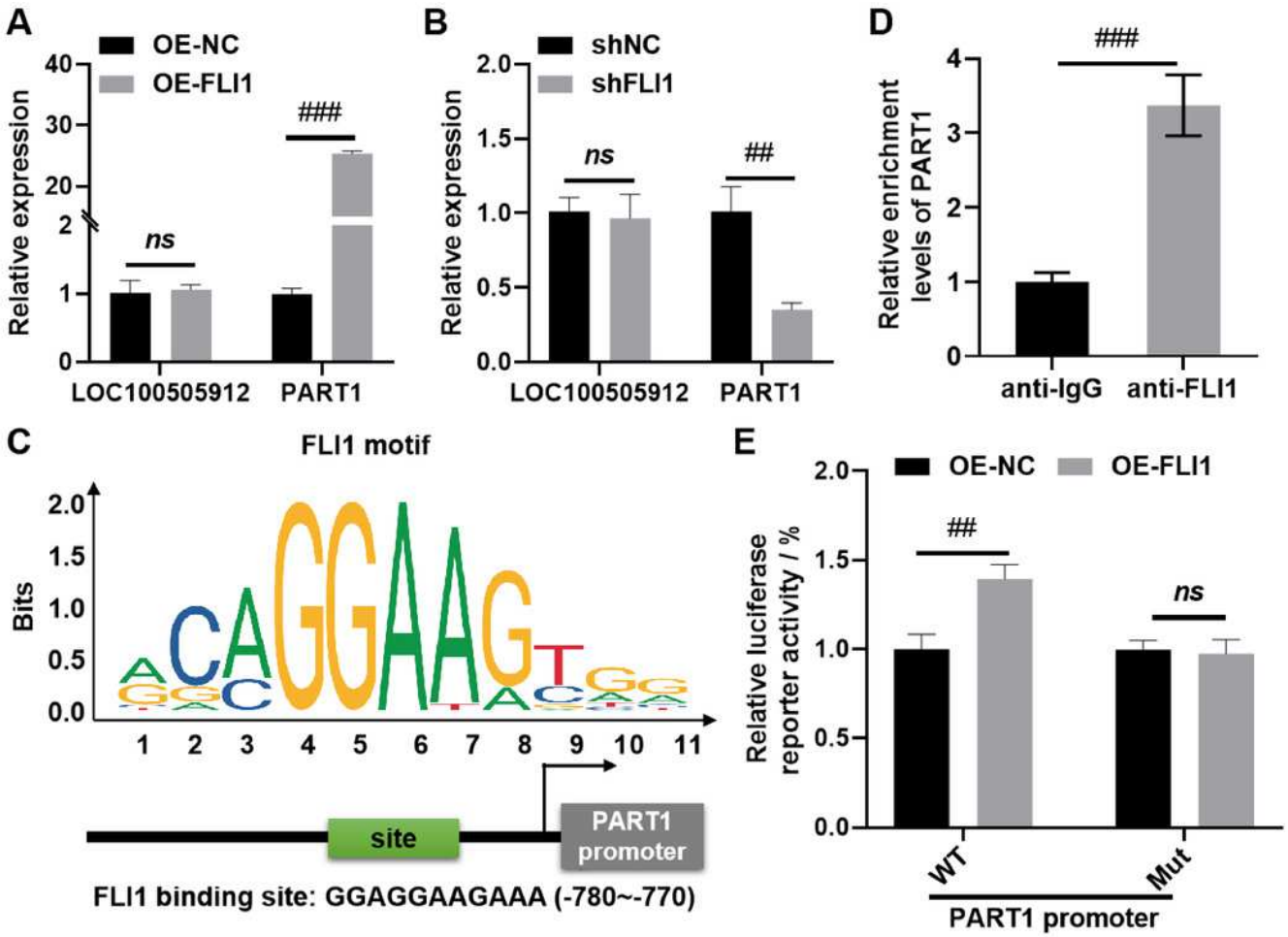
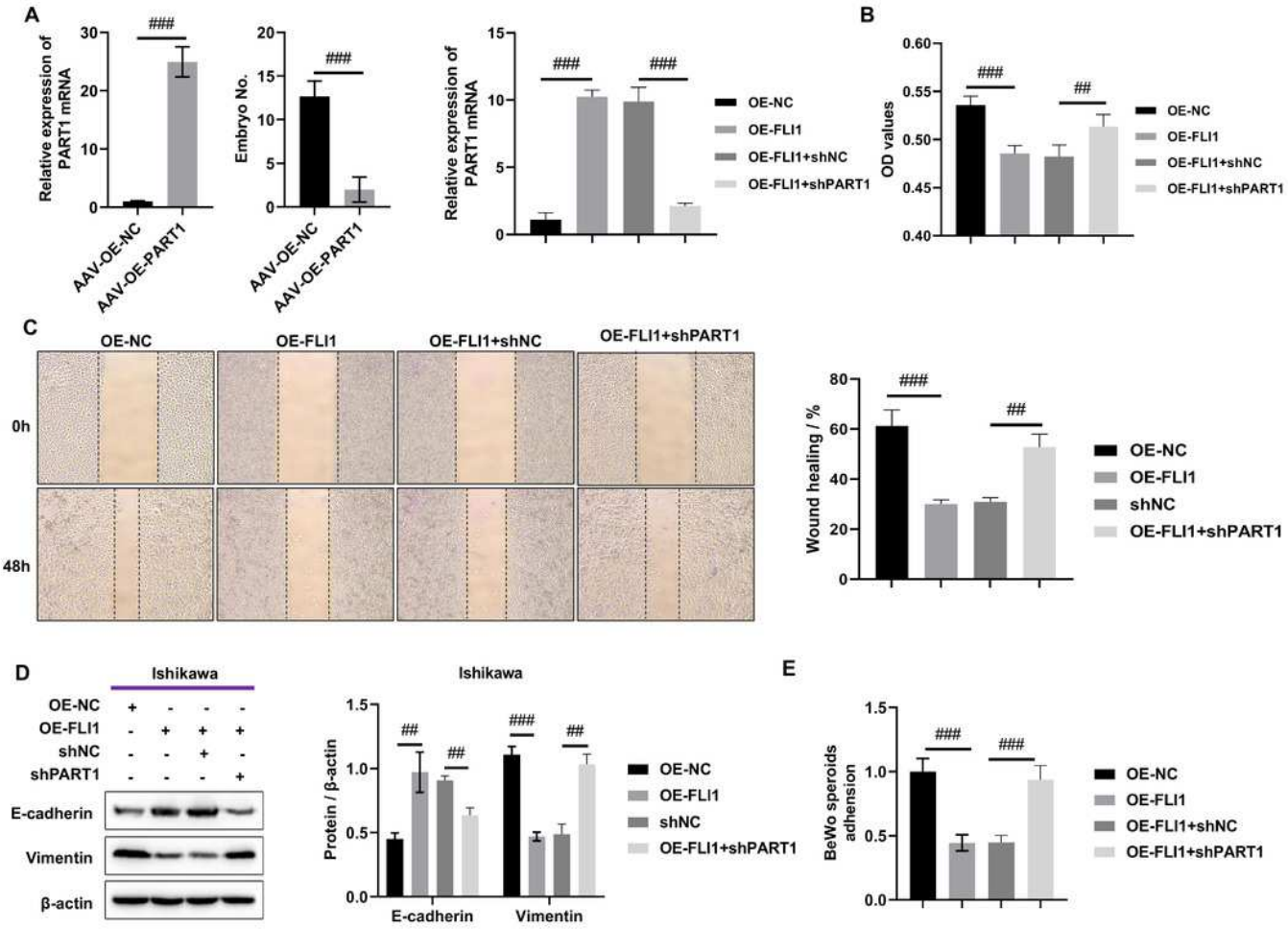


Figure 7

Effects of epithelial cell specific overexpression of PART1 on mouse embryo implantation ability and endometrial epithelial cell function.

(A) The mRNA expression level of PART1 in mouse endometrial epithelial cells and the effect of overexpression of PART1 on the number of embryo implantation in pregnant mice; (B) Expression level of PART1 in endometrial epithelial cells and detection of endometrial epithelial cell proliferation in each group using CCK-8 method; (C) Scratch test to detect cell invasion ability; (D) Western blot assay for detecting the expression changes of Vimentin protein and E-cadherin protein in Ishikawa cells; (E) BeWo cell ball adhesion test to detect changes in adhesion ability. AVV-OE PART1 and AVV-OE-NC: PART1 overexpress adeno-associated viruses and their negative control adeno-associated viruses; OE-FLI1 and OE-NC: FLI1 overexpression plasmids and their negative control plasmids; ShPART1 and shNC: PART1 silencing plasmid and its negative control plasmid; ## $P<0.01$, ### $P<0.001$.


A
Relative expression of PART1 mRNA
###
AAV-OE-NC
AAV-OE-PART1
Embryo No.
###
AAV-OE-NC
AAV-OE-PART1
Relative expression of PART1 mRNA
###
###
OE-NC
OE-FLI1
OE-FLI1+shNC
OE-FLI1+shPART1
B
OD values
###
##
OE-NC
OE-FLI1
OE-FLI1+shNC
OE-FLI1+shPART1
C
OE-NC
OE-FLI1
OE-FLI1+shNC
OE-FLI1+shPART1
0h
48h
Wound healing / %
###
##
OE-NC
OE-FLI1
shNC
OE-FLI1+shPART1
D
Ishikawa
OE-NC
OE-FLI1
shNC
shPART1
E-cadherin
Vimentin
β-actin
Ishikawa
Protein / β-actin
##
##
###
##
E-cadherin
Vimentin
OE-NC
OE-FLI1
shNC
OE-FLI1+shPART1
E
BeWo speroids adhension
###
###
OE-NC
OE-FLI1
OE-FLI1+shNC
OE-FLI1+shPART1