SI Appendix

Deciphering the endometrial niche of human thin endometrium at single-cell resolution

Haining Lv a,1, Guangfeng Zhao a,1, Peipei Jiang a, Huiyan Wang a, Zhiyin Wang a, Simin Yao a, Zhenhua Zhou a, Limin Wang b, Dan Liu a, Wenbo Deng c, d,2, Jianwu Dai e,2, Yali Hu a,2.

This word file includes:

Materials and Methods

Figure S1 to 9

Table S1 to 6
Materials and Methods

Sample collection

Human endometrial samples and procedures involved in this study were approved by the Ethics Committee of Nanjing Drum Tower Hospital (No.2016-129-01). Informed written consent was obtained from each participant before the endometrial biopsy was performed. Forty-five patients who needed hysteroscopic examination at the Infertility Consulting Clinic at Nanjing Drum Tower Hospital were enrolled in this study, including twenty patients with thin endometria and twenty-five controls with normal endometria. The diagnosis of thin endometrium was based on their endometrial thickness<7mm at mid-luteal phase or had histories of embryos transfer cancellation in vitro fertilization procedures due to thin endometrium. Those patients also presented scanty menstruation, poor response to estrogen stimulations and normal uterine cavity by hysteroscopy. The normal controls had normal menstrual blood volume and the endometrial thickness was between > 8mm -<14mm at mid-luteal phase and normal ovary function. All participants presented regular menstrual cycling, normal karyotype and negative serological tests for human immunodeficiency virus, hepatitis B virus, hepatitis C virus and syphilis. Patients with endometriosis, leiomyoma or adenomyosis or polycystic ovary syndrome were excluded. The samples of endometria were taken in uterine body near to the fundus under the hysteroscopic guidance at late proliferating phase of the participants' natural menstrual cycles(1). The late proliferating phase was defined based on follicle size between 15 and 18mm by ultrasonography and a low level of serum progesterone. Among 45 participants, three thin endometria and three normal controls were carried on scRNA-seq and the demographic characteristics of them were included in SI Appendix, Table S1. Five normal endometrial samples from controls were used for isolating endometrial stromal cells and uterine natural killer cells (uNKs) for the following cell culture and treatment. Other endometrial samples were used for the mRNA and protein extraction, immunohistochemistry, immunofluorescence and flow cytometry. The clinical information of the other 39 patients was listed in SI Appendix, Table S3.

Isolation of endometrial cells

Endometrium was rinsed several times with PBS until without obvious blood clots; then was cut into small pieces, which were digested with 0.1% Trypsin (Wisent Inc., Canada) for 8min and then transferred into collagenase 0.8mg/mL Type I (Sigma-Aldrich, USA) for 60min at 37°C, 5% CO2, with regular vigorous shaking in a humidified incubator. The released endometrial cells were filtered through 70μm cell strainer (BD Biosciences, USA), centrifuged and re-suspended in 5mL of red blood cell lysis buffer (eBiosciences, USA) for 8min to exclude any remaining red blood cells. Finally, the endometrial cells were re-suspended in PBS and then were used for single cell 3’-cDNA library preparation followed by 10X Genomics Chromium Single Cell 3’reagent Kits protocol. Single cell encapsulation, complementary DNA (cDNA) library synthesis and RNA-sequencing were completed by Gene Denovo (Guangzhou, China).
Single cell RNA-seq data processing

Single cell libraries were sequenced on Illumina NovaSeq instruments using 150 nt paired-end sequencing. Reads were processed using the Cell Ranger 4.0.0 pipeline with the default and recommended parameters. FASTQs generated from Illumina sequencing output were aligned to the human reference genome (GRCh38) using the STAR algorithm. Next, Gene-Barcode matrices were generated for each individual sample by counting unique molecular identifiers (UMIs) and filtering non-cell associated barcodes. Finally, the gene-barcode matrix containing the barcoded cells and gene expression counts were generated.

This output was then imported into the Seurat (v3.0) R toolkit for quality control and downstream analysis of our single cell RNA-seq data. All functions were run with default parameters, unless specified otherwise. Cells that expressed fewer than 200 genes or mitochondrial gene content > 15% of the total UMI count were excluded. Before incorporating a sample into our merged dataset, we individually inspected the cells-by-genes matrix of each as a Seurat object.

Identification of cell types by Uniform Manifold Approximation and Projection (UMAP)

The Seurat package implemented in R was applied to identify major cell types. Highly variable genes were generated and used to perform PCA. Significant principal components were determined using JackStraw analysis and visualization of heatmaps focusing on PCs 1 to 30. PCs 1 to 30 were used for graph-based clustering (at res = 0.3) to identify distinct groups of cells.

Cluster markers identification

The cluster-specific marker genes were identified by running the FindAllMarkers function in Seurat package to normalize gene expression data. To identify differentially expressed genes between two clusters, `find.markers` function was applied. The R package clusterProfiler was utilized to perform biological process enrichment analysis with the top 20 differentially expressed genes in each cluster or subset. Cell analysis was done with CellCycleScoring function in Seurat package with embedded S and G2/M phase genesets.

Cell-cell communication analysis

To investigate potential interactions across different cell types in the endometrial cell, cell-cell communication analysis was performed using CellPhoneDB, which is a publicly available repository of curated receptors and ligands and their interactions. Enriched receptor–ligand interactions between two cell types were derived based on the expression of a receptor by one cell type and the expression of the corresponding ligand by another cell type. To identify the significant cell–cell interaction, we performed permutation tests between two cell types mediated by a specific ligand–receptor pair based on the mean gene expression of ligand from one cell type and the corresponding receptor from another cell type, and \( p \)-value < 0.01 was considered statistically significant.
To further analyze and compare the intercellular communications difference between thin and normal endometrial samples, CellChat, an open source R package (https://github.com/sqjin/CellChat), was employed for thin and normal endometrium scRNA-seq data. First, we inferred intercellular communications among stromal and other cell subsets for thin and normal datasets separately, and then, analyzed them together via joint manifold learning and classification of the inferred communication networks based on their functional similarity.

**Cell culture**

Human endometrial stromal cells (hESCs) were isolated from normal endometrial samples at late proliferating phase. The method for endometrial cell dissociation was described above. hESCs were obtained from endometrial cells by being filtering through 40\(\mu\)m cell strainers to eliminate glandular clumps. Then they were cultured in DMEM/F12 media with 10% fetal bovine serum (FBS) (Gibco, Australia). The medium was changed every 2 to 3 days according to the growth of the cells. Cells from passages 2-4 were used for all experiments. Decidualization was induced in hESCs as described by Brosen(2). hESCs were cultured in DMEM/F12 containing 2.5% FBS and treated with 0.5 mM 8-Br-cAMP (B7880, Sigma-Aldrich) and 1\(\mu\)M methoxyprogesterone acetate (MPA) (M1629, Sigma-Aldrich) for 72 h.

THP1 and Jurkat cells were recovered and cultured in RPMI-1640 media with 10% FBS. THP1 cells were differentiated into macrophage by 5ng/mL PMA (Sigma-Aldrich, USA) for 48 h. Cells were treated with specified compounds in accordance with the experimental parameters indicated. Time-matched vehicle controls ensured the effects were mediated by independent variables. The proteins and inhibitions used for cell treatment in this paper were listed in SI Appendix, Table S4.

**Isolation and culture of uNK cells**

The digested endometrial cells mentioned above were re-suspended in 5mL of red blood cell lysis buffer for 8min to exclude any remaining red blood cells. Finally, the endometrial cells were suspended to stain CD56-PE (BD Biosciences, 1:50), CD45-PE-cy7 (Biolegend, 1:50) and Fixable Viability Stain 510 (FVS510, BD Biosciences, 1:100) for fluorescence-activated cell sorting (FACs) in order to isolate uNKs. Then acquired uNK were cultured in RPMI-1640 media with 10% FBS and 2ng/mL IL-15 to aid uNK cell maturation and never used beyond 7 days in culture.

**Fluorescent-activated cell sorting for perivascular cells**

In brief, human endometrial tissues were cut into small pieces and digested with 0.1% Trypsin (Wisent Inc., Canada) for 8 min and then transferred into Type I collagenase (0.8mg/mL, Sigma-Aldrich, USA) for 60min at 37°C, 5% CO2, with regular vigorous shaking in a humidified incubator. The released endometrial cells were filtered through 70\(\mu\)m cell strainer (BD Biosciences, USA), centrifuged and re-suspended in 5mL of red blood cell lysis buffer (eBiosciences, USA) for 8 min to exclude any remaining red blood cells. Then the endometrial
cells were re-suspended in PBS and stained with FVS510 (BD Biosciences, 1:100), CD31-APC-cy7 (Biolegend, 1:50) and SUSD2-PE (Biolegend, 1:50) for 20 min at room temperature. Then perivascular cells (FVS510-CD31-SUSD2+) were sorted using FACS Aria III with FACS Diva software (BD Biosciences) and cultured in DMEM/F12 medium containing 10%FBS in 5% CO2 at 37°C.

RNA extraction and reverse-transcriptase quantitative polymerase chain reaction (RT–qPCR) analyses

For conventional RNA analysis, total RNA from the tissues or cultured cells was extracted with Trizol reagent (Invitrogen Life Technologies, USA). 1μg of RNA was reverse transcribed into cDNA using HiScript® III RT Super Mix for qPCR (+gDNA wiper) (Vazyme, China). Individual qPCR mixes were made according to the recommendations of the manufacturer of ChamQ SYBR® qPCR Master Mix (Without ROX) (Vazyme, China). Differences among the target gene expression levels were estimated by the ΔΔCt method and normalized to the level of 18S. The primers used in this study were listed in SI Appendix, Table S5.

Western blotting

Tissues or cells were lysed in lysis buffer (Biosharp, China) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (MedChemExpress, USA) and centrifugated for 15 min at 12,000 rpm. The protein concentration of cell lysates was determined by Pierce BCA protein assay kit (Thermo Scientific, USA). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. Blots were visualized by ECL. GAPDH was used as loading control. The antibodies used in this study were listed in SI Appendix, Table S6.

Immunohistochemistry

Endometrial tissues were fixed with 4% phosphate-buffered paraformaldehyde overnight, and then dehydrated and embedded in paraffin. Paraffin sections (2μm) were prepared, dewaxed, hydrated, and the endogenous peroxides were quenched with 3% H2O2. After heat-mediated antigen retrieval, the slides were incubated with antibodies for 2h at 37°C. After incubation with prediluted HRP-conjugated secondary antibodies (Typing, China), the sections were exposed to DAB and counterstained with hematoxylin. Leica DM6B microscope was used for visualization. The antibodies used in this paper were listed in SI Appendix, Table S6.

Immunofluorescence

Tissues from thin endometrium and the controls were fixed with 4% phosphate-buffered paraformaldehyde overnight, dehydrated in graded sucrose and then embedded in OCT and finally frozen. The cryostat sections were incubated in blocking buffer (1% bovine serum albumin, 0.1% Triton-X in PBS) at room temperature for 30min. Primary and secondary antibodies were added, followed by being stained with DAPI. The antibodies used in this paper
Flow cytometry

Biopsies of thin endometrium and the controls for flow cytometry were obtained and dissociated following the protocol used for the 10x dataset. Then, cells were re-suspended in PBS. To examine immune cell subsets, cells were stained for viability (FVS510, BD Biosciences, 1:100) and the following antibodies: CD45-PE-Cy7 (Biolegend, 1:50), CD56-BV421 (BD Biosciences, 1:50), CD3-PE (eBiosciences, 1:50) for 20min at room temperature. Cells were washed twice with PBS, then resuspended in 100uL PBS. Data was acquired using CytoFLEX (Beckman Coulter, USA). Analysis was performed using CytExpert (Beckman Coulter, USA).

Cell proliferation and cell cycle analysis

Cell proliferation was analyzed using a CCK-8 kit (Dojindo Molecular Technologies, Japan). After hESCs being treated for 48 h, CCK-8 was added separately to each well and incubated for an additional 2h. The absorbance at 450nm was measured using a Multiskan GO microplate spectrophotometer (Thermo Scientific, USA). Cell cycle analysis was performed after 48 h treatment. Briefly, the cells were fixed in cold 75% ethanol and incubated overnight at 4 °C. After being washed twice with PBS, the cells were incubated with 50μg/mL PI, 50μg mL/mL RNase A and 0.1% Triton X-100 for 30min at 4 °C and detected by CytoFLEX. The data was analyzed with ModFit software.

Masson staining

Endometrial tissues of suitable size were collected and dehydrated in 70, 80, 90, and 100% ethanol and transparentized in dimethylbenzene. After being-embedded in paraffin, tissues were cut into slices. The slices were dewaxed, hydrated, incubated in Weigert solution for 5-10min. They were then differentiated in acidic ethanol for 5-15s, slightly washed with water, and blued in Masson bluing buffer for 3-5min. After being washed with water, the slices were incubated in ponceau-fuchsin solution for 5-10min, washed with weak acid solution for 1min, and washed with phosphomolybdic acid solution for 1-2min. The slices were subsequently stained in aniline blue solution for 1-2min. They were then washed with weak acid solution, dehydrated in absolute ethanol, transparentized in dimethylbenzene, mounted with neutral resin, and observed under a microscope.

Colony formation assay

Perivascular cells from normal and thin endometrium were seeded in 6-well in 5% CO₂ at 37°C. After culturing for 48 h, non-adherent cells were discarded and adherent cells were washed twice with PBS. Growth medium was replaced with fresh medium for every 3 days. After 2 weeks, colonies were stained with 0.1% crystal violet solution and counted under the inverted microscope.

Statistical Analyses

The experimental data were statistically analyzed using two-tailed t test to compare differences.
between different groups and treatments with or without Welch's correction depending on the variance of data with PRISM software (GraphPad 8 Software). ANOVA with a Tukey post hoc test was used for multiple comparisons. Data were presented as means ± standard error of mean (SEM). *P value < 0.05 was considered statistically significant. In all figures, one, two and three asterisks indicate *P < 0.05, **P < 0.01 and ***P < 0.001, respectively; NS indicates not significant.

The bioinformatics data was statistically analyzed using a two-tailed t test with R language, and P values were indicated in each figure. The boxplot represented the median, the first quartile and the third quartile of values and the whisker represented 1.5 times the interquartile distance. Data in bar plots were shown as the mean ± SEM.

Reference

1. Song M, et al. (2021) circPTPN12/miR-21-5 p/Np63alpha pathway contributes to human endometrial fibrosis. Elife 10: e65735.
2. Brosens JJ, Hayashi N, & White JO (1999) Progesterone receptor regulates decidual prolactin expression in differentiating human endometrial stromal cells. Endocrinology 140(10):4809-4820.
**Fig. S1** Distribution of each sample in normal and thin endometrium and expressions of marker genes in normal endometrium. (A) UMAP of cell types in different samples. (B) Distribution of cell markers PTPRC, PDGFRB, COL4A1, SUSD2 and PROX1 by UMAP in normal endometrium. (C) Representative images of immunofluorescence for CD45, Ki67, CD31, PDGFRB, COL4A1, SUSD2 and PROX1 in normal endometrium (n=5). Scale bars, 100μm.
Fig. S2 Gene expression pattern in normal endometrial cells. (A) The expression pattern of highly expressed genes in Str compared to pStr and Peri analyzed using the TCSeq package in R. Expression of these genes was normalized to Z Score and the color indicated the membership values representing the degree of genes belonging to this cluster. (B) The functional enrichment of highly expressed genes in Str compared to pStr and Peri. (C) Genes related to cell cycle that were highly expressed in pStr. (D) Expression of RGS5 and NOTCH3 which was specifically located in Peri by UMAP. (E) Genes related to IL-17 signaling pathway.
lysosome and protein processing in ER that were highly expressed in GE. (F)

Immunofluorescence staining for AXIN2, CK7, pH3, CD163, CD8 and CD56 in normal endometrium (n=5). Scale bars, 100 μm.
Fig. S3 The characterization of gene expression patterns in thin endometrium compared to normal. (A) Expression and distribution of POLR3K in normal and thin endometrium by violinplot and UMAP. Data were presented as mean ± SEM. *P<0.05. (B) Expression of NME4 in normal and thin endometrium by UMAP. (C) mRNA expression levels of CDKN2A between normal and thin endometrium examined by RT–qPCR (n=12, per group). Data were presented as mean ± SEM. *P<0.05. (D) Immunohistochemical staining for p16 and p21 in normal and thin endometrium at proliferative phase (n=5, per group). Scale bars, 100 μm. Data were presented as mean ± SEM. *P<0.05, ***P<0.001. (E) Western blot analysis for p21, PTN and collagen1 in normal and thin endometrium (n=7, per group). (F) mRNA expression levels of PTGS2 between normal and thin endometrium examined by RT–qPCR (n=10, per group). Data were presented as mean ± SEM. *P<0.05.
**Fig. S4** Over-deposition of collagen in thin endometrium. (A) Distribution of COL4A1 in normal and thin endometrium by UMAP and expression of COL4A1 in Str, pStr and Peri by violinplot. Data were presented as mean ± SEM. ***P<0.001. (B) Location of CD31 and COL4A1 in normal and thin endometrium by immunofluorescence (n=5, per group). Data were presented as mean ± SEM. ***P<0.001. (C) Expression of VCAN and MGP in Str, pStr and Peri by violinplot. Data were presented as mean ± SEM. ***P<0.001. (D) Masson staining for normal and thin endometrium at proliferating phase (n=5, per group). Scale bars, 100 μm.
**Fig. S5** Cell connection in endometrium at proliferating phase. The outgoing signaling and incoming signaling among different cell types in normal (A) and thin (B) samples analyzed by CellphoneDB. Cell types marked by red had the obvious changes in numbers of interaction pairs. Cell cycle and CCK-8 analysis for hESCs after treatment with the supernatants of THP1-derived macrophage (THP1) (C-D), Jurkat (E-F) and uNK (G-H) for 48 h, respectively. Data were presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.
Fig. S6 The role of EGF downstream signal pathway in endometrium growth. (A) Quantitative analysis of p-ERK, p-STAT3, p-S6 and p-AKT expression in endometrium examined by immunohistochemical staining (n=5, per group). Data were presented as mean ± SEM. **P<0.01, ***P<0.001. (B) The effects of signaling inhibitors for AKT (3CAI or LY294002), ERK (PD98059 or U0126), mTOR (PF-4708671 or rapamycin) and STAT3 (cryptotanshinone or S3I) on their downstream as confirmed by western blot. Cell cycle analysis for hESCs after treatment with AKT inhibitor 3CAI (C) or LY294002 (D), ERK inhibitor PD98059 (E) or U0126 (F), mTOR inhibitor PF-4708671 (G) or rapamycin (H) and STAT3 inhibitor cryptotanshinone (I) or S3I (J) for 48 h, respectively.
The effect of hESCs decidualization at the present of different signaling pathway inhibitors. The AKT inhibitors 3CAI (A) or LY294002 (B), ERK inhibitors PD98059 (C) or U0126 (D), mTOR inhibitors PF-4708671 (E) or rapamycin (F), STAT3 inhibitors cryptotanshinone (G) or S3I (H), IHH inhibitor cyclopamine (I) and recombinant IHH protein (J) were added in the culture medium of hESCs respectively, followed by 8-Br-cAMP (8-Br) and methoxyprogesterone acetate (MPA) for 72 h. Expression of decidualization markers PRL and IGFBP1 was examined by RT–qPCR. Data were presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.
Fig. S8 The role of IHH, SEMA3B, PTN and TWEAK in the growth of endometrium. (A-B) Cell cycle analysis for hESCs after treatment with cyclopamine or IHH for 48 h, respectively. (C) Quantitative analysis of SEMA3B and PTN expressions in endometrium examined by
immunohistochemical staining (n=5, per group). Data were presented as mean ± SEM. **P<0.01. (D) Cell cycle analysis for hESCs after treatment with recombinant SEMA3B for 48 h. (E) Distribution of PTN in normal and thin endometrium by UMAP. (F) mRNA expression level of PTN between normal and thin endometrium examined by RT–qPCR (n=10, per group). Data were presented as mean ± SEM. *P<0.05. (G) Cell cycle and CCK-8 analysis for hESCs after treatment with PTN protein for 48 h. Data were presented as mean ± SEM. *P<0.05, **P<0.01. (H) The effect of PTN siRNA (siPTN) on inhibiting PTN expression confirmed by western blot, and cell cycle and CCK-8 analysis for hESCs after treatment with siPTN for 48 h. Data were presented as mean ± SEM. *P<0.05, ***P<0.001. (I) Circle plot showing the inferred TWEAK signaling networks among different cell types in normal endometrium. (J) Immunohistochemical staining for TNFSF12 in normal and thin endometrium. Scale bars, 100 μm (n=5, per group). (K) Cell cycle and CCK-8 analysis for hESCs after treatment with TNFSF12 protein for 48 h. Data were presented as mean ± SEM. **P<0.01, ***P<0.001.
Fig. S9 The effect of decidualization of hESCs after treatment with SEMA3B, PTN and TNFSF12. The proteins of SEMA3B (A), PTN (B) and TNFSF12 (C) were added in the culture medium of hESCs respectively, followed by 8-Br-cAMP (8-Br) and methoxyprogesterone acetate (MPA) for 72 h. Expression of decidualization markers PRL and IGFBP1 was examined by RT–qPCR. Data were presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.
Table S1 Clinical characteristics of women included in scRNA-seq analysis

|                      | NC-1 | NC-2 | NC-3 | Thin-1 | Thin-2 | Thin-3 |
|----------------------|------|------|------|--------|--------|--------|
| Number of cells      | 7679 | 9826 | 11959| 9998   | 7137   | 9827   |
| Age (year)           | 29   | 31   | 27   | 28     | 30     | 31     |
| Times for D&C        | 0    | 0    | 0    | 3      | 2      | 2      |
| Times for TCRA       | 0    | 0    | 0    | 3      | 1      | 2      |
| Maximum EM (mm)      | 9.4  | 8.9  | 11.8 | 4.1    | 6.2    | 5.5    |
| Menstrual blood volume | normal | normal | normal | scant spotting | oligomenorrhea | scant spotting |
| Times of embryo transfer cancellation | -    | -    | -    | -      | 3 (EM=5.1, 6.2, 6.2 mm before ET) | 2 (EM=6.1, 5.9 mm before ET) |
| Length of menstrual cycle | 28   | 30   | 30   | 30     | 29     | 30     |
| Day after menstruation | 13   | 13   | 14   | 14     | 14     | 14     |
| Follicle size on the day of hysteroscopy | 18*18*17 | 17*16*17 | 16*16*18 | 17*18*16 | 18*18*18 | 17*16*16 |
| EM on the day of hysteroscopy | 8.4  | 8.1  | 9.7  | 3.2    | 4.5    | 3.8    |
| Serum progesterone   | 0.34 | 0.14 | 0.21 | 0.23   | 0.27   | 0.23   |

a) D&C, dilation and curettage; TCRA, transcervical resection of adhesion; EM, endometrial thickness; ET, embryo transfer.
Table S2 Cell numbers and proportions of normal and thin endometrium in scRNA-seq analysis

| cell type  | NC cell number | percentage in total | Thin cell number | percentage in total |
|------------|----------------|---------------------|------------------|---------------------|
| 1 B_cell   | 56             | 0.19%               | 39               | 0.14%               |
| 2 CD4_T    | 667            | 2.27%               | 441              | 1.63%               |
| 3 CD8_T    | 1434           | 4.88%               | 1062             | 3.93%               |
| 4 Cili_Epii| 269            | 0.92%               | 356              | 1.32%               |
| 5 Endo     | 775            | 2.64%               | 1064             | 3.94%               |
| 6 GE       | 1156           | 3.94%               | 1527             | 5.65%               |
| 7 LE       | 1413           | 4.81%               | 1098             | 4.06%               |
| 8 Lymph    | 64             | 0.22%               | 40               | 0.15%               |
| 9 Macro    | 879            | 2.99%               | 759              | 2.81%               |
| 10 NK      | 1270           | 4.32%               | 629              | 2.33%               |
| 11 NK_T    | 91             | 0.31%               | 177              | 0.66%               |
| 12 Peri    | 1392           | 4.74%               | 1645             | 6.09%               |
| 13 pNK     | 20             | 0.07%               | 45               | 0.17%               |
| 14 pStr    | 2285           | 7.78%               | 1801             | 6.67%               |
| 15 Str     | 17602          | 59.93%              | 16329            | 60.45%              |
| Total      | 29373          | 100.00%             | 27012            | 100.00%             |
**Table S3 Patient clinical information**

| Items                             | Control (n = 22) | Thin endometrium (n = 17) | \( P \) value |
|-----------------------------------|------------------|---------------------------|---------------|
| Age (year)                        | 29.35±0.40       | 29.61±0.71                | > 0.05        |
| Times for D&C                     | 0.35±0.12        | 2.2±0.33                  | < 0.001       |
| Times for TCRA                    | 0                | 1.11±0.23                 | -             |
| Maximum endometrial thickness (mm)| 11.39±0.35       | 4.89±0.23                 | < 0.001       |
| Times of embryo transfer cancellation | 0              | 2.44±0.26                 | -             |
| Length of menstrual cycle         | 30.52±0.43       | 29.39±0.34                | > 0.05        |
| Day after menstruation            | 13.08±0.23       | 12.61±0.27                | > 0.05        |
| Serum progesterone                | 0.62±0.07        | 0.58±0.11                 | > 0.05        |

b) D&C, dilation and curettage; TCRA, transcervical resection of adhesion.
| Protein or inhibition | Catalog   | Manufacturer          | working concentration |
|-----------------------|-----------|-----------------------|-----------------------|
| PTN                   | 252-PL    | R&D system            | 100ng/mL              |
| TNFSF12               | P6048     | Abnova                | 30ng/mL               |
| SEMA3B                | 9518-S3   | R&D system            | 100ng/mL              |
| 3CAI                  | HY-16666  | MedChemExpress        | 4μM                   |
| LY294002              | HY-10108  | MedChemExpress        | 10μM                  |
| PD98059               | HY-12028  | MedChemExpress        | 10μM                  |
| U0126                 | 662005    | MERCK                 | 5μM                   |
| PF-4708671            | HY-15773  | MedChemExpress        | 200nM                 |
| rapamycin             | HY-10219  | MedChemExpress        | 100nM                 |
| cryptotanshinone      | HY-N0174  | MedChemExpress        | 5μM                   |
| S3I                   | 473102    | MERCK                 | 100μM                 |
| Indian Hedgehog       | 1705-HH   | R&D system            | 100ng/mL              |
| cyclopamine           | HY-17024  | MedChemExpress        | 10μM                  |
| Gene name | Forward Primer (5’-3’) | Reverse Primer (5’- 3’) |
|-----------|------------------------|------------------------|
| 18S       | CTTTGTCGCTCCTCCTC      | CTGACCGGGTTGTTTTGAT    |
| PTN       | GGAGCTGAGTGCAAGCAAAC   | CTCGCTTCAGACTTCCAGTTTC|
| POLR2I    | GGCTTCGTGGGTATTCCTT    | CTCCTGCTGGTAATCACAGTTTC|
| NM4       | AGGGTACAATGTTCGCCG     | GACGCTGAAGTCACCCCTTAT  |
| PTGS2     | CGACGTCCGCTGCATACAG    | CGCCTTATACTGGTCAAATCCC|
| MKI67     | ACGGCCTGTTACTATCAAAAGG | CAGACCCATTATCTTGTGTTGGA|
| CDKN2A    | ATGGAGCCTTCGCTGACT     | GTAACATTTCGGTGCTTGAGG  |
| Antigen      | Label | Host species | Clone       | Catalog   | Manufacturer | Application | Reference       |
|--------------|-------|--------------|-------------|-----------|--------------|-------------|-----------------|
| CD45         | -     | Rabbit       | ab10558     | Abcam     | IF           | Fig. 1F      |
| CD45         | -     | Mouse        | MEM-28      | ab8216    | Abcam        | IF           | Fig. 1D, Fig. S1C|
| CD14         | -     | Rabbit       | SP192       | ab183322  | Abcam        | IF           | Fig. 1F, Fig. 3G |
| TBR2/Eomes   | -     | Rabbit       | EPR21950-241| ab216870  | Abcam        | IF           | Fig. 3G         |
| NCAM1        | -     | Mouse        | 123C3       | 3576      | Cell Signaling Technology | IF | Fig. 1F, Fig. S2F |
| GNLY         | -     | Rabbit       | EPR22110-101| ab241333  | Abcam        | IF           | Fig. 1F         |
| CD4          | -     | Rabbit       | EPR6855     | ab133616  | Abcam        | IF           | Fig. 3G         |
| CD8          | -     | Mouse        | C8/144B     | ab17147   | Abcam        | IF           | Fig. 1F, Fig.S2F |
| PR           | -     | Mouse        | Alpha PR6   | ab2765    | Abcam        | IF           | Fig. 3G         |
| CD10         | -     | Mouse        | 56C6        | ab951     | Abcam        | IF           | Fig. 1K         |
| Axin2        | -     | Rabbit       |             | ab32197   | Abcam        | IF           | Fig. S2F        |
| WT1          | -     | Rabbit       | CAN-R9(IHC)-56-2 | ab89901 | Abcam        | IF           | Fig. 1D         |
| HAND2        | -     | Rabbit       | EPR19451    | ab200040  | Abcam        | IF           | Fig. 1D         |
| PDGFR beta   | -     | Rabbit       | Y92         | ab32570   | Abcam        | IF           | Fig. S1C        |
| PROX1        | -     | Rabbit       | EPR19273    | ab199359  | Abcam        | IF           | Fig. S1C        |
| PTN          | -     | Goat         | AF-252-PB   | R&D Systems | IHC         | Fig. S3F     |
| Semaphorin 3B| -     | Rabbit       | NBP2-94522  | Novus     | IHC          | Fig. 7F      |
| TWEAK        | -     | Rabbit       |             | ab37170   | Abcam        | IHC/IF       | Fig. S8J        |
| Collagen IV alpha 1 | - | Rabbit | ab189408 | Abcam | IF | Fig. S1C, 4B |
| Phospho-Histone H3 | - | Rabbit | D7N8E | 53348 | Cell Signaling Technology | IF | Fig. 1K, Fig. S2F |
| Ki67         | -     | Rabbit       | ab15580     | Abcam     | IF           | Fig. S1C     |
| CD31         | -     | Rabbit       | ab28364     | Abcam     | IF           | Fig. S1C     |
| CD31         | -     | Mouse        | JC/70A      | ab9498    | Abcam        | IF           | Fig. S1C        |
| Antibody/CODD  | Species | Clone | Catalog No. | Vendor       | Type       | Fig. |
|----------------|---------|-------|-------------|--------------|------------|------|
| SUSD2          | Mouse   | W5C5  | 327401      | Biolegend    | IF         | S1C  |
| CK7            | Mouse   | RCK105| ab9021      | Abcam        | IF         | S1C  |
| p16            | Rabbit  | ZM-0205| ZSGB-BIO    | IHC          | S3E        |
| p21            | Rabbit  | EPR362| ab109520    | Abcam        | IHC/WB     | S3E  |
| Phospho-S6 Ribosomal Protein | Rabbit | D57.2.2E | 4858 | Cell Signaling Technology | IHC/WB | 5D   |
| S6 Ribosomal Protein | Mouse | 54D2  | 2317 | Cell Signaling Technology | IHC/WB | 5D   |
| Phospho-Erk1/2 | Rabbit | D13.14.4E | 4370 | Cell Signaling Technology | IHC/WB | 5D   |
| Erk1/2         | Rabbit  | 137F5 | 4695        | Cell Signaling Technology | IHC/WB | 5D   |
| Phospho-Stat3  | Rabbit  | D3A7  | 9145        | Cell Signaling Technology | IHC/WB | 5D   |
| Stat3          | Mouse   | 124H6 | 9139        | Cell Signaling Technology | IHC/WB | 5D   |
| Phospho-Akt    | Rabbit  | D9E   | 4060        | Cell Signaling Technology | IHC/WB | 5D   |
| Akt            | Rabbit  | C67E7 | 4691        | Cell Signaling Technology | IHC/WB | 5D   |
| PTCH1          | Rabbit  | ab53715| Abcam      | IHC/WB       | 6D   |
| Collagen Type I | Rabbit | 14695-1-AP | Proteintech | WB          | S3E  |
| GAPDH          | HRP     | AC035 | Abclonal    | WB           |      |
| Goat anti-rabbit IgG | HRP | 7074 | Cell Signaling Technology | WB |      |
| Goat anti-mouse IgG | HRP | 7076 | Cell Signaling Technology | WB |      |
| Goat anti-rabbit IgG | Alexa Fluor 488 | 111-545-003 | Jackson ImmunoResearch | IF |      |
| Goat anti-mouse IgG | Alexa Fluor 488 | 115-545-003 | Jackson ImmunoResearch | IF |      |
| Goat anti-rabbit IgG | Rhodamine | 111-025-003 | Jackson ImmunoResearch | IF |      |
| Goat anti-mouse IgG | Rhodamine | 115-025-003 | Jackson ImmunoResearch | IF |      |