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Single-cell transcriptomic landscape of the mid-secretory eutopic endometrium reveals receptivity defects in adenomyosis

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Abstract

Background Adenomyosis (AM) is a common gynecological disorder in reproductive-age women, with impaired endometrial receptivity as a key contributor to infertility. However, a comprehensive single-cell atlas of the eutopic endometrium during the implantation window remains lacking, particularly regarding the roles of specific subpopulations in shaping the implantation microenvironment. This gap has limited mechanistic insights into AM-associated implantation failure.

Methods We performed 10x Genomics single-cell RNA sequencing of mid-secretory eutopic endometrium from three primary infertile AM patients and three parous controls, validated by qRT-PCR, immunohistochemistry, and immunofluorescence.

Results AM-derived cells accounted for a higher proportion of epithelial, perivascular, and proliferative populations, whereas stromal and immune cell fractions remained largely unchanged. Notably, an expanded epithelial subpopulation characterized by the stem cell markers *LGR5* and *SOX9* exhibited excessive proliferative activity and strengthened tight junctions, collectively disrupting the establishment of a receptive epithelial phenotype. In the stroma, a *DIO2*⁺ decidual subpopulation with senescence-associated secretory phenotype (SASP) features was markedly reduced in AM, accompanied by downregulation of inflammatory mediators and key decidualization markers, indicative of impaired decidualization. Cell–cell communication analysis revealed marked remodeling of epithelial–stromal crosstalk in AM. Hyperactive epithelial PTN and loss of ncWNT could be linked to aberrant proliferative activity and polarity alterations, while reduced epithelial DKK1 and IGF2 signaling, together with stromal ncWNT loss, may partially explain deficient decidualization.

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Conclusions In summary, our findings indicate that excessive proliferation and impaired differentiation of epithelial cells, along with insufficient inflammatory activation and decidualization defects in stromal cells, jointly underlie reduced endometrial receptivity in AM. Moreover, altered epithelial signaling may further compromise stromal decidualization.

Keywords Adenomyosis, Endometrial receptivity, Single-cell RNA sequencing.

Introduction

Adenomyosis (AM) is a common gynecological disorder in women of reproductive age, characterized by the infiltration of active endometrial glands and stroma into the myometrium, often accompanied by myometrial hyperplasia, hypertrophy, and fibrosis. Clinically, AM not only causes debilitating symptoms such as dysmenorrhea and menorrhagia, which substantially impair quality of life, but is also strongly associated with infertility and recurrent pregnancy loss [1]. The prevalence of AM has been reported to be 24.4% among infertile women, increasing to 38.2% in those with recurrent miscarriage and to 34.7% in women with a history of pregnancy failure [2]. In assisted reproductive technology cycles, including in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI), AM patients exhibit lower clinical pregnancy rates and higher miscarriage rates compared with women of reproductive age in the general population [3]. Notably, embryo quality remains unaffected, highlighting that impaired endometrial receptivity is likely the predominant cause of implantation failure in AM [4].

Endometrial receptivity refers to a series of morphological and molecular changes within the endometrium during the window of implantation (WOI) that create a favorable environment for embryo attachment and invasion. The luminal epithelium, lining the uterine cavity, represents the first maternal cell population to contact the embryo. Its differentiation status during the WOI is a decisive determinant of implantation success. In the receptive phase, epithelial cells must undergo a transition from a proliferative to a secretory phenotype, marked by microvilli reduction, loss of apical–basal polarity, loosening of intercellular junctions, and increased secretory activity, thereby facilitating blastocyst adhesion and invasion [5]. Incomplete execution of this transformation can act as a physical barrier, impeding implantation efficiency. Decidualization of endometrial stromal cells (ESCs) is another essential event in establishing receptivity. In the mid-secretory phase, ESCs respond to rising progesterone and cAMP levels by activating the transcription factor forkhead box O1 (FOXO1), which drives decidual transformation [6]. During this process, ESCs differentiate into either mature decidual cells or acutely senescent decidual cells (snDCs) [7]. The latter secrete a spectrum of senescence-associated secretory phenotype (SASP) factors that transiently shape the local inflammatory milieu, promote a short-lived “window

of inflammation” conducive to implantation, and help maintain maternal–fetal immune tolerance and decidual homeostasis [8]. Insufficient decidualization or functional impairment of decidual cells is strongly linked to implantation failure [9, 10]. Coordinated communication between epithelial and stromal cells is indispensable for achieving synchronized receptivity [11]. For example, the progesterone-induced epithelial signal molecule Indian Hedgehog (IHH) promotes stromal decidualization by upregulating COUP-TFII expression in ESCs [12, 13], whereas stromal cells secrete fibroblast growth factors (FGFs), WNT ligands, and insulin-like growth factor 1 (IGF1) that feedback to regulate epithelial differentiation and function [14, 15]. These bidirectional signaling loops collectively ensure structural integrity and temporal coordination of the receptive endometrium. Disruption of this finely tuned epithelial–stromal crosstalk may impair embryo–endometrium communication and lead to implantation failure.

Previous studies have linked impaired receptivity in AM to a range of pathological changes, including structural and functional alterations at the endometrial–myometrial junction, immune dysregulation, abnormal angiogenesis, oxidative stress, and disruption of implantation-related signaling pathways [16]. Moreover, aberrant expression of key implantation molecules—such as integrins, leukemia inhibitory factor (LIF), homeobox A10 (HOXA10), and components of the WNT and Hedgehog pathways—has been reported in AM, suggesting defective signaling during the acquisition of receptivity [3, 17]. Most previous studies [18, 19] have focused on the impact of individual molecules on receptivity, which, while informative, cannot capture the complexity of the endometrial microenvironment or reveal novel cellular subpopulations and signaling mechanisms. Single-cell RNA sequencing (scRNA-seq) offers a systematic approach to overcome these limitations by mapping cellular heterogeneity and intercellular communication at high resolution [20]. While several scRNA-seq studies have explored the pathogenesis of AM [21–23], these efforts largely examined ectopic lesions or eutopic endometrium during the proliferative phase, thereby emphasizing disease initiation rather than implantation failure. By contrast, endometrial receptivity is established exclusively during the mid-secretory phase, making it the clinically most relevant window for implantation. To date, no study has generated a single-cell atlas of the eutopic

Table 1 Baseline characteristics of scRNA-seq patients in AM and control groups

	Control (n = 3)	AM (n = 3)	P value
Age (year), median (IQR)	35.00 (33.50–35.50)	34.00 (33.00–34.50)	0.80
BMI, median (IQR)	20.03 (19.87–21.46)	21.17(20.49– 21.49)	1.00
Endometrial thickness (mm) on LH + 7, median (IQR)	8.00 (6.65–9)	10.00 (9.00–10.50)	0.50
Live birth, median (range)	1 (0–1)	0 (0–0)	0.40
Menstrual cycling	Regularly (6–7 days every 28–30 days)	Regularly (6–7 days every 28–30 days)	

Table 2 Clinical characteristics of the validation cohort

	Control (n = 12)	AM (n = 12)	P value
Age (year)	31.25 ± 2.93	32.50 ± 2.20	0.25
BMI, median	21.95 ± 1.80	21.25 ± 1.52	0.31
Basal FSH (mIU/mL)	6.54 ± 1.29	6.28 ± 1.49	0.66
Basal LH (mIU/mL)	3.87 ± 1.04	3.95 ± 0.97	0.85
Basal E2 (pg/mL)	38.12 ± 13.47	42.95 ± 12.14	0.37
Endometrial thickness (mm) on LH + 7	8.54 ± 0.74	8.52 ± 0.46	0.92
Times of embryo transfers (range)	1 (1, 1)	2 (1, 4)	< 0.0001
Median number of embryo transfers (range)	1 (1, 1)	3 (1, 6)	< 0.0001
Median score of transferred day-3 embryos (range)	8 (7, 10)	8 (7, 10)	0.92
Live birth, median (range)	1 (0–1)	0 (0–1)	0.01
Menstrual cycling	Regularly (6–7 days every 28–30 days)	Regularly (6–7 days every 28–30 days)	

Data were presented as mean ± SD and were analyzed by Student's t-test, except for the times of embryo transfers, the number of embryo transfers, score of transferred day-3 embryos and live birth calculated using the independent samples Mann–Whitney U test (median, range). BMI, body mass index; FSH, follicle stimulating hormone; LH, luteinizing hormone; E2, estradiol

endometrium in AM during this phase, leaving a critical gap in understanding the cellular and molecular basis of reduced receptivity.

In this study, we leveraged scRNA-seq to construct a cellular atlas of the eutopic endometrium during the WOI in AM patients compared with healthy controls. Our analyses primarily focused on epithelial and stromal compartments that are closely associated with implantation, and provided a preliminary characterization of their altered signaling interactions. These findings offer insights into the cellular basis of impaired receptivity in AM.

Materials and methods

Patients and sample collection

This study was approved by the Institutional Ethics Committees of Ruijin Hospital (KY2020–3) and Shanghai Ninth People's Hospital (SH9H–2020-TK6-1), Shanghai Jiao Tong University School of Medicine. Written informed consent was obtained from all participants. Endometrial samples from women with primary infertility due to adenomyosis (AM group, *n* = 3) were collected during the mid-secretory phase at the Center for Reproductive Medicine, Ruijin Hospital. Control endometrial tissues (*n* = 3), obtained from women with a history of fertility but with infertility caused by tubal obstruction or male factors, were collected at the Department of Assisted Reproduction, Shanghai Ninth People's Hospital. Patient demographic and clinical characteristics are presented in Table 1. Single-cell RNA sequencing (scRNA-seq) was performed for all enrolled samples. For validation, additional mid-secretory endometrial tissues were obtained from 12 women with adenomyosis and 12 controls meeting identical inclusion criteria (Table 2). The diagnosis of adenomyosis was established based on clinical symptoms in combination with imaging findings from transvaginal ultrasonography (TVUS) and magnetic resonance imaging (MRI). TVUS criteria included diffuse uterine enlargement, asymmetric myometrial thickening, heterogeneous myometrial echotexture, and an indistinct endometrial–myometrial junction. MRI diagnostic indicators comprised a junctional zone thickness > 12 mm and high-signal intensity foci within the myometrium on T2-weighted images. Exclusion criteria included the use of intrauterine devices or hormonal contraceptives within the preceding three months; presence of endocrine or metabolic disorders (e.g., polycystic ovary syndrome, diabetes mellitus, insulin resistance, thyroid dysfunction); chromosomal abnormalities in either partner; coexisting endometriosis, uterine fibroids, hydrosalpinx, intrauterine adhesions, or congenital uterine malformations; thrombophilia; autoimmune diseases; and a body mass index (BMI) > 24 kg/m². All tissues were collected on LH + 7 using a sterile endometrial suction curette (LILYCLEANER, Shanghai, China).

Single-cell dissociation

Fresh endometrial tissues were immediately rinsed with ice-cold phosphate-buffered saline (PBS) to remove residual blood and mechanically minced into approximately 1 mm³ fragments. The fragments were enzymatically digested with collagenase type IV (1 mg/mL; Sigma-Aldrich, USA) at 37 °C for 15–20 min with gentle agitation. The resulting suspension was filtered through a 70-μm cell strainer (Falcon, USA), and cells were collected by centrifugation at 400 × *g* for 7 min. Red blood cells were removed using lysis buffer (Solarbio, Beijing,

China) for 15 min on ice, followed by washing in PBS containing 0.04% bovine serum albumin (BSA). The cell suspension was subsequently passed through a 35- μ m filter (Falcon, USA) to eliminate debris. Cell viability was determined by acridine orange/propidium iodide (AO/PI) staining with a Countstar Fluorescence Cell Analyzer, and only samples with viability >90% were included. To further enrich viable cells, a Dead Cell Removal Kit (Miltenyi Biotec, Germany) was applied according to the manufacturer's instructions.

scRNA-seq data processing

We utilized 10 $\times$ Genomics scRNA-seq technology to profile individual cells from the 6 tissue samples. The sequence data were aligned to the hg38 human genome for quality control and gene quantification using Cell Ranger (v.7.1.0) with default parameters. It is generated gene-cell sparse matrices for each sample by Cell Ranger software. We integrated and analyzed the data from all 6 patients, including downloaded from published data as control group samples using standard pipeline of the Seurat package (v4). In brief, we combined the count matrices from all the samples into a single merged matrix. We retained cells meeting the following quality criteria: (1) expressed 500 and 5,000 genes; (2) cells with UMI count less than 50,000; (3) cells with mitochondrial gene expression percentages fewer than 25. Genes expressed in fewer than 5 cells were filtered out to avoid excluding rare cell types. Next, the filtered gene expression matrix was normalized and log-transformed using 'NormalizeData' and 'ScaleData' function from Seurat package. Highly variable genes were identified with 'FindVariableFeatures' function and used to perform principal component analysis (PCA) with $n_comps=20$. Then we applied the mutual nearest neighbors (MNN) algorithm for batch correction to integrate the different samples (Supplementary Fig. 1), and the batch-corrected principal components were used for further analyses, including nearest-neighbor graphs. Finally, we performed unsupervised clustering using the Leiden algorithm on the neighborhood graph generated by 'FindNeighbors' and 'FindClusters' function.

To address cell cycle heterogeneity, we first obtained a cell cycle gene list and calculated cell cycle scores for each cell using the CellCycleScoring function in Seurat. Clusters were categorized into major cell lineages based on the average expression of well-established marker genes: stromal cells (*DCN*+ and *PDGFRA*+), epithelial cells (*KRT8*+, *KRT18*+ and *WFDC2*+), immune cells (*PTPRC*+), vascular cells (*VASC*, *ACTA2*+ and *VWF*+), and proliferative cells (*MKI67*+ and *TOP2A*+). Within the stromal and endothelial cell clusters, sub-clusters were further delineated and annotated as specific subtypes based on the expression of relevant gene

sets in each major cell type. Identification of differentially expressed genes were using 'FindMarkers' function in Seurat. Genes with p-adjusted value (FDR) < 0.05 (adjusted using the Benjamini–Hochberg (BH) procedure) and log fold change > 0.25 and minimum percent of expressed genes > 0.1 were considered significantly upregulated in a given cluster or sub-cluster. Enrichment analysis of biological process Gene Ontology (GO) terms for these differentially expressed genes was performed using the 'enrich' function from R package ClusterProfiler (v4.0.1). Signaling pathway activity scores were calculated and Z-score normalized across samples, allowing comparison of relative pathway activities between groups.

Cell-type proportion analysis

For each sample, cell-type proportions were calculated as the number of cells in each cluster divided by the total cell count. Enrichment or depletion of specific cell types was assessed by the log₂ ratio of observed to expected proportions, with expected values derived from total cluster sizes across samples.

Pseudotime analysis

Cell differentiation trajectories were reconstructed using Monocle2 [24]. The top 1,000 variable genes with FDR < 0.05 after BH correction were used to order cells along pseudotime. Gene variability was assessed using log₂ (counts + 1)-transformed data and calculated by 'differentialGeneTest' function, and trajectories were inferred with default Monocle2 parameters.

Cell–cell communication analysis

Intercellular signaling was evaluated using CellChat (v1.6.1) and NicheNet (v1.1.1). Overexpressed ligands and receptors in each cell group were identified and mapped to known human ligand–receptor interaction databases. Protein–protein interaction networks were incorporated to refine pathway inference. To ensure statistical rigor, ligand–receptor interactions were filtered using the Benjamini–Hochberg (BH) false discovery rate (FDR) procedure, and only interactions with p-adjusted value (FDR) < 0.05 were retained. Additionally, only interactions involving ≥ 10 cells per group were considered for downstream analysis.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and reverse transcribed with PrimeScript™ RT Master Mix (Takara, Japan). Quantitative PCR was performed using SYBR Green Master Mix (Takara) on an Applied Biosystems 7500 Real-Time PCR System (USA). Primer sequences are listed in Supplementary Table 1. GAPDH was used as an internal control, and relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$

method. All reactions were conducted in triplicate, and experiments were repeated at least three times.

Immunofluorescence (IF) staining

Endometrial tissues and cultured cells were fixed in 4% paraformaldehyde at 4 °C overnight. Tissues were cryoprotected in 30% sucrose, embedded in OCT, and stored at −80 °C. Sections (15 µm) were cut using a Leica cryostat, air-dried at 45 °C for 40 min, and rinsed in PBS. Sections were treated with 0.3 mol/L glycine, permeabilized, and blocked with 0.5% Triton X-100 in 3% BSA/PBS for 4 h. Primary antibodies (Supplementary Table 2) were applied overnight at 4 °C, followed by washing and incubation with fluorophore-conjugated secondary antibodies for 2–3 h at room temperature.

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded tissue sections (5 µm) were deparaffinized, rehydrated, and subjected to antigen retrieval. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min. Sections were blocked with 5% goat serum for 30 min and incubated with primary antibodies at 4 °C overnight, followed by secondary antibody incubation. Signal was developed with diaminobenzidine (DAB) and counterstained with hematoxylin. Antibodies are listed in Supplementary Table 2.

Enzyme-linked immunosorbent assay (ELISA)

Endometrial tissues and cell culture supernatants were processed for ELISA analysis. Endometrial tissues were homogenized in PBS containing protease inhibitors, sonicated, and centrifuged to obtain supernatants. Cell culture supernatants were harvested and clarified by centrifugation to remove debris. Concentrations of prolactin (PRL, Multisciences, China), insulin-like growth factor-binding protein 1 (IGFBP1, Multisciences, China), C-X-C motif chemokine ligand 14 (CXCL14, Mlbio, China), tissue inhibitor of metalloproteinases 3 (TIMP3, Multisciences, China), and Interleukin 15 (IL15, Multisciences, China) were determined following manufacturers' protocols. Optical density at 450 nm was measured using a microplate reader.

Cell culture

Primary human endometrial epithelial cells (HEECs) and stromal cells (HESCs) were isolated as previously described [25]. Endometrial tissues were digested with 1 mg/mL type IV collagenase (Gibco, USA) at 37 °C for 1 h and sequentially filtered through 100-µm and 40-µm strainers to obtain stromal and epithelial fractions, respectively. Cells were cultured in DMEM/F12 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Dcell, China), penicillin (100 U/mL, NCM, China), and

streptomycin (0.1 mg/mL, NCM, China) at 37 °C with 5% CO₂.

Small interfering RNA (siRNA) knockdown and plasmid overexpression

Primary HEECs and HESCs were transfected at 50–60% confluence. Control, LGR5, SOX9, and DIO2 overexpression plasmids (GeneCopoeia, China) were introduced using X-tremeGENE 9 (Roche, USA). Gene-specific siRNAs targeting DIO2, LGR5, or SOX9, or a scrambled control siRNA (15 nM; GenePharma, China), were transfected using Lipofectamine RNAiMAX (Invitrogen, USA) according to the manufacturer's instructions. siRNA sequences are listed in Supplementary Table 3.

Cell proliferation assays

Cell proliferation of HEECs was evaluated using the Cell Counting Kit-8 (CCK-8; Yeasen, China) following the manufacturer's protocol. Transfected cells were seeded into 96-well plates at a density of 2,000 cells per well and cultured for the indicated time points. CCK-8 reagent (10 µL) was added to each well and incubated at 37 °C for 1 h, after which absorbance at 450 nm was measured using a microplate reader.

Cell proliferation was examined using the BeyoClick EdU-488 assay (Beyotime, China) in accordance with the manufacturer's protocol. Transfected cells cultured in 48-well plates were incubated with EdU to label proliferating cells, followed by nuclear counterstaining with DAPI. Fluorescence images were acquired using an inverted fluorescence microscope (Axio Vert.A1, Zeiss, Germany) from three randomly selected fields per well.

Western blotting

Cells were lysed in RIPA buffer (Epizyme, China) supplemented with protease inhibitors (NCM, China). Protein concentrations were quantified using a BCA assay (Epizyme, China), and equal amounts of protein were resolved by SDS-PAGE (10%) and transferred onto 0.22 µm PVDF membranes (Millipore, USA). Membranes were blocked with 5% non-fat milk and incubated overnight at 4 °C with primary antibodies, followed by HRP-conjugated secondary antibodies. Protein bands were detected using ECL reagents (Yeasen, China) and quantified by densitometry with ImageJ software, normalized to GAPDH. Antibody details are provided in Supplementary Table 2.

In vitro decidualization

Primary HESCs were isolated from mid-secretory endometrium of AM patients and controls, then transfected with siRNAs or plasmids for 48 h. For decidualization, the transfected cells were grown in red-free DMEM/F12 (Gibco) with 2% charcoal stripped FBS (CS-FBS, Dcell)

in the presence of 1 μ M medroxyprogesterone acetate (MPA) (MCE, China) and 0.5 mM 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) (Sigma, USA).

Dickkopf-related protein 1 (DKK1) and inhibitor treatment

Primary HEECs and HESCs were isolated from normal mid-secretory endometrium. To modulate epithelial WNT signaling, HEECs were treated with DKK1 (100 ng/mL; MCE) or a DKK1 inhibitor (antiDKK1, 1 μ M; MCE). Culture supernatants were subsequently collected, centrifuged to remove cellular debris, and used as epithelial-conditioned medium. This conditioned medium was applied to HESC cultures to evaluate epithelial–stromal paracrine interactions. Following conditioned medium exposure, HESCs were induced to undergo in vitro decidualization with MPA and 8-Br-cAMP as described above.

Senescence-associated β -galactosidase (SA- β -gal) staining

SA- β -gal activity was detected using a staining kit (Beyotime, China). Cells were fixed at room temperature for 15 min, washed three times with PBS, and incubated with the staining solution at 37 °C in a CO₂-free environment overnight. Senescent cells were identified by blue-green staining and imaged under a light microscope the next day.

Statistical analysis

Continuous variables were compared between two groups using a two-tailed Student's t-test or, when appropriate, nonparametric tests. For multiple-group comparisons, one-way ANOVA with Bonferroni post hoc correction was applied to normally distributed data; otherwise, the Kruskal–Wallis test was used. Categorical variables were analyzed using Fisher's exact test. Statistical analyses were performed using GraphPad Prism (v9.5.1; GraphPad Software Inc., USA), and a P-value < 0.05 was considered statistically significant.

Results

Single-cell transcriptomics of mid-secretory eutopic endometrium in adenomyosis

To comprehensively characterize the cellular composition and transcriptional landscape of the endometrium, we collected mid-secretory phase samples from adenomyosis patients (AM; $n = 3$) at Ruijin Hospital and retrieved control samples ($n = 3$) from the GEO database (GSE183837) for single-cell transcriptomic profiling. ScRNA-seq libraries from AM samples were constructed using the 10x Genomics Chromium platform (Fig. 1A). After quality control and normalization, 30,027 high-quality single cells were retained for downstream analyses, including 15,148 from AM and 14,879 from controls. The median sequencing depth reached 8,800 reads per cell, with a median of 2,708 genes detected. Cells were

clustered based on canonical marker gene expression and visualized by UMAP (Fig. 1B), identifying five major cell types. Representative marker gene expression patterns are shown in heatmaps (Figs. 1C and D): stromal cells (Str; 84.05%, expressing *DCN* and *PDGFRA*), epithelial cells (Epi; 2.90%, expressing *KRT8*, *KRT18* and *WFDC2*), immune cells (8.10%, expressing *PTPRC*), vascular cells (VASC; 3.08%, expressing *ACTA2* and *VWF*), and proliferative cells (1.87%, expressing *MKI67* and *TOP2A*). To evaluate the robustness of our cell-type and subpopulation definitions, we performed sensitivity analyses using random subsampling strategies. Specifically, we randomly selected 60% and 80% of cells from the full dataset and repeated unsupervised clustering analyses (Supplementary Fig. 2). Importantly, when compared with clustering results obtained from 100% of cells, the key cell subpopulations and their defining marker genes were consistently preserved, supporting the stability of our clustering strategy. As expected, stromal cells represented the most abundant population, constituting the majority of the functional layer of the endometrium [26]. Although the overall cellular yield and major lineage composition were comparable between the AM and control groups (Fig. 1E), the relative contributions of AM and control cells within individual cell types were markedly altered (Fig. 1F). Specifically, epithelial, VASC, and proliferative cell numbers were elevated in AM relative to controls, with epithelial cells showing the most pronounced increase, whereas stromal and immune cell numbers remained largely unchanged. Previous studies have reported that in the basal layer of eutopic endometrium from AM patients, aberrant epithelial proliferation and enhanced angiogenesis provide structural and nutritional support for lesion formation and invasion [27]. Our data extend these observations by demonstrating that abnormal expansion of specific cell subsets also occurs within the functional layer during the mid-secretory phase in AM. These findings indicate pathological remodeling within the functional layer of eutopic endometrium in AM, thereby potentially contributing to impaired endometrial receptivity.

Stem cell-like LGR5 SOX9 epithelial cells drive hyperproliferation in the mid-secretory eutopic endometrium of adenomyosis

As the critical interface for blastocyst implantation, the endometrial epithelium plays a central role in establishing uterine receptivity and transmitting signals to other tissue compartments [28]. Single-cell transcriptomic analysis of eutopic endometrium revealed a marked enrichment of epithelial cells in AM compared with controls, warranting further investigation. Based on canonical marker gene expression profiles, four epithelial subclusters (Epi1–Epi4) were identified (Fig. 2A and B). Epi1

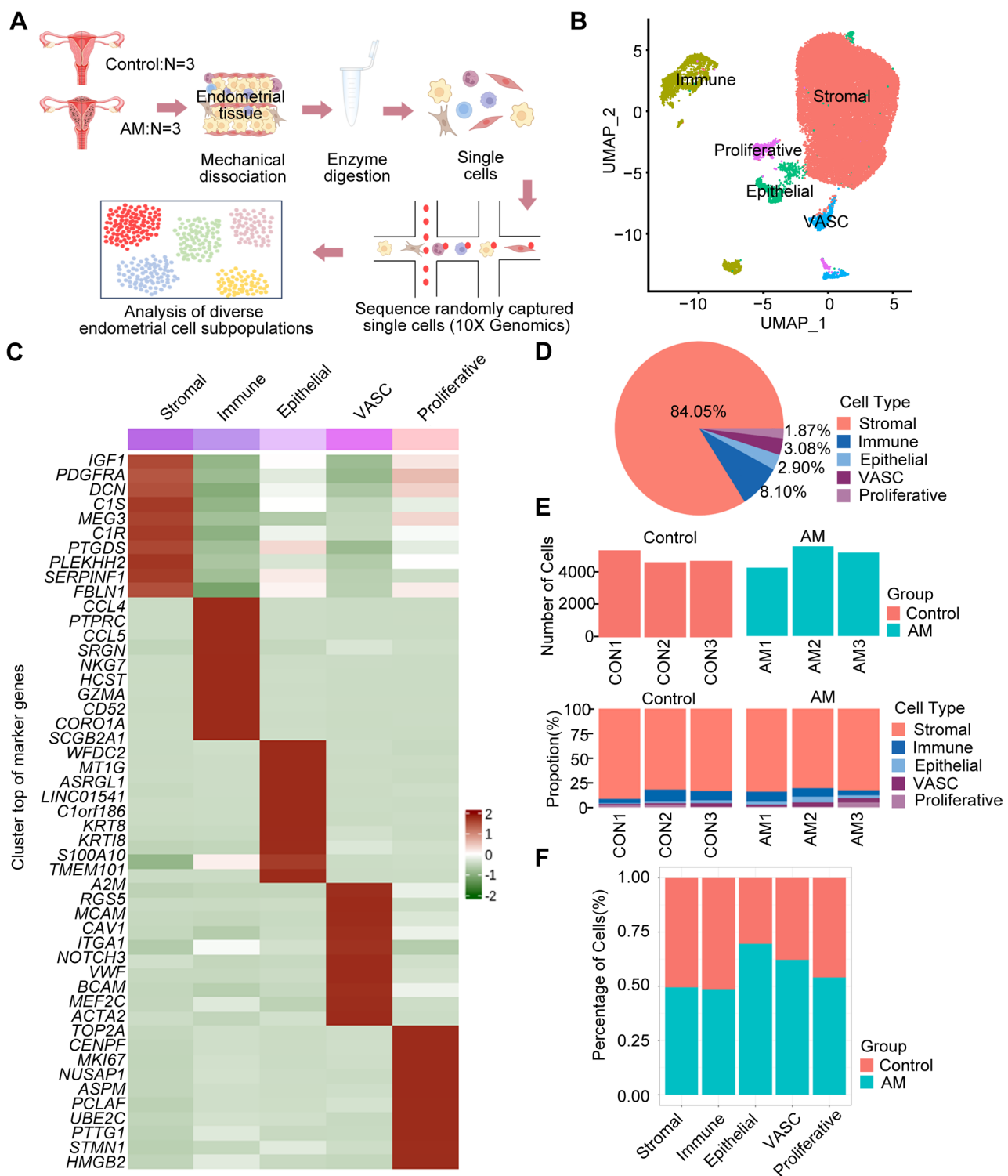


Fig. 1 Single-cell transcriptomic profiling of mid-secretory eutopic endometrium from adenomyosis (AM) patients and controls. **(A)** Workflow of sample collection and single-cell RNA sequencing (scRNA-seq). Endometrial tissues from AM patients (n=3) and controls (n=3) during the window of implantation were processed for scRNA-seq using the 10x Genomics Chromium platform. **(B)** UMAP of all captured single cells, colored by the five major cell types: stromal, epithelial, immune, vascular (VASC), and proliferative cells. **(C)** Heatmap showing the expression patterns of representative marker genes for each major cell type. **(D)** A pie chart depicting the overall proportions of stromal, epithelial, immune, VASC, and proliferative cells in the endometrium across all samples. **(E)** Stacked bar plots illustrating total cell numbers and the distribution of stromal, epithelial, immune, VASC, and proliferative cells within each individual sample. **(F)** Box plots showing the relative proportions of AM and control cells within each cell type

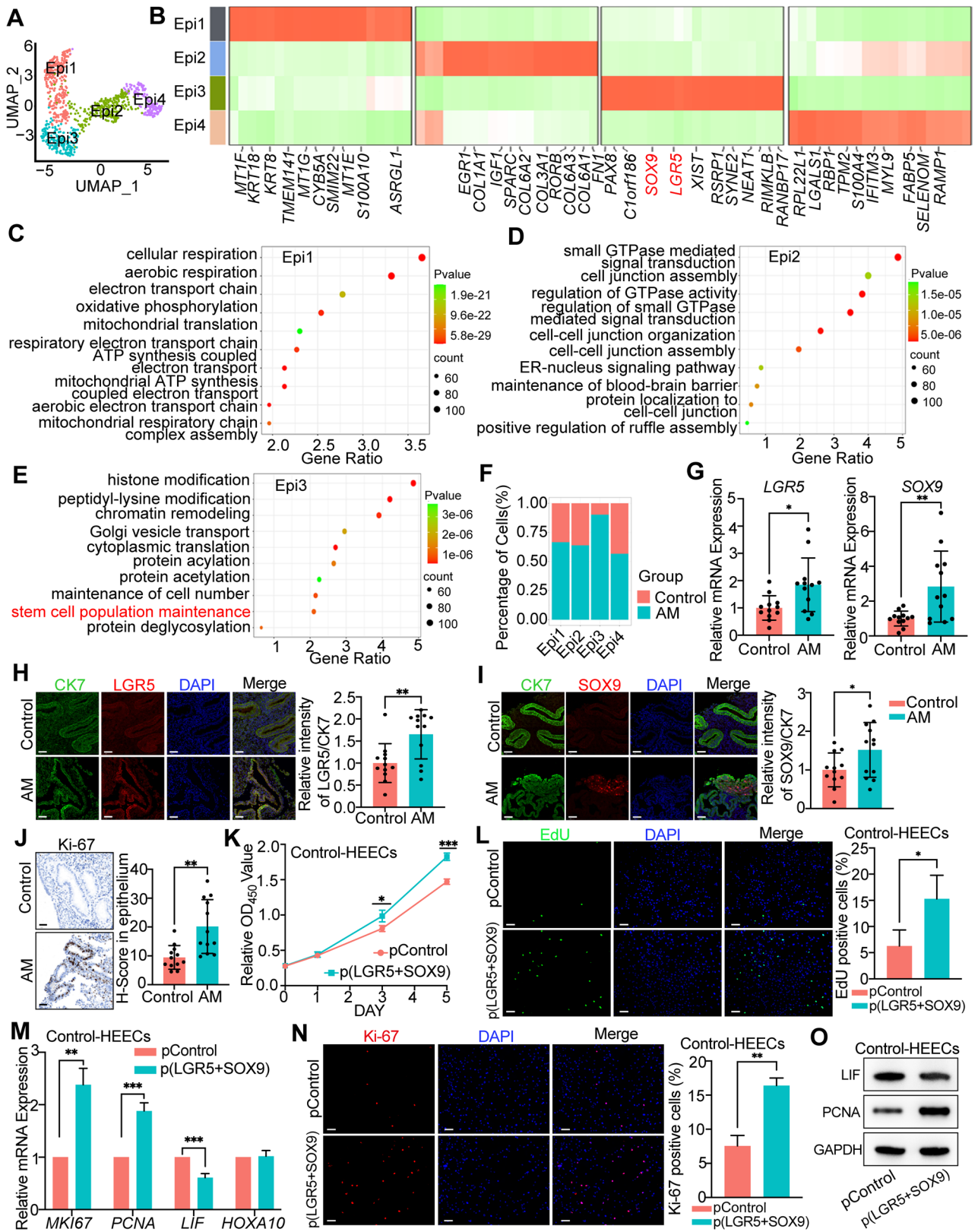


Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 Characterization of eutopic endometrial epithelial cells in adenomyosis (AM). **(A)** UMAP plot showing four epithelial cell subclusters (Epi1–Epi4) **(B)** Heatmap of the top marker genes for each epithelial subcluster. **(C–E)** Gene Ontology (GO) enrichment analyses of top marker genes in Epi1 **(C)**, Epi2 **(D)**, and Epi3 **(E)**. **(F)** Box plots showing the relative proportions of AM and control cells within each epithelial subcluster. **(G)** LGR5 and SOX9 mRNA expressions in mid-secretory eutopic endometrium from AM and control patients examined by qPCR analysis ($n = 12$ per group). **(H, I)** Immunofluorescence staining for the expression and localization of LGR5 and SOX9. Scale bars, 50 μm ; $n = 12$ per group. **(J)** Immunohistochemical staining for the expression of proliferation marker Ki-67. Scale bars, 50 μm ; $n = 12$ per group. **(K)** Cell proliferation following plasmid LGR5 and SOX9 (p(LGR5 + SOX9)) overexpression in human endometrial epithelial cells (HEECs) isolated from mid-secretory endometrial tissues of controls ($n = 3$) measured by CCK-8 assay. **(L)** Proliferation of HEECs transfected with p(LGR5 + SOX9) determined via EdU assay. Actively proliferating cells are visualized by green fluorescence, with all nuclei counterstained in blue. Scale bar = 100 μm ; $n = 3$. **(M)** RT-qPCR quantification of proliferation markers (*MKI67*, *PCNA*) and differentiation markers (*LIF*, *HOXA10*) levels after LGR5 and SOX9 overexpression in control HEECs ($n = 3$). **(N)** Representative immunofluorescence images for Ki-67 (red) and corresponding quantification of positive cells in HEECs. Scale bar = 100 μm ; $n = 3$. **(O)** Western blotting of LIF and PCNA protein levels in HEECs transfected with p(LGR5 + SOX9). Data are presented as the mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$

expressed classical epithelial markers (*KRT18* and *KRT8*) together with high levels of metallothionein-1 family genes (*MT1F*, *MT1E* and *MT1G*). Functional enrichment analysis indicated activation of “oxidative phosphorylation” and “ATP synthesis” (Fig. 2C). Consistent with previous bulk [29] and single-cell transcriptomic studies [30], metallothioneins are involved in proliferation, repair, and antioxidant responses, serving as markers of the proliferative-to-early-secretory transition. Within Epi1, the AM group accounted for a significantly larger fraction of cells than controls (Fig. 2F), indicating that many epithelial cells in AM persist in an incompletely differentiated state during the mid-secretory phase. Epi2 was characterized by high expression of extracellular matrix (ECM) and collagen synthesis genes (*COL3A1*, *COL1A1*, *COL6A1*, *FN1*) (Fig. 2B), with enrichment in “cell–cell junction” and “ER–nucleus signaling pathway” functions (Fig. 2D). Normally, progesterone-mediated loosening of junctions and suppression of estrogen signaling enhance receptivity during this phase [31]. In Epi2, AM-derived cells were more abundant (Fig. 2F), reflecting reinforced epithelial junctions and sustained estrogen signaling in AM, which may hinder implantation window progression. Notably, the Epi3 subcluster exhibited high expression of stem cell markers leucine-rich repeat containing G protein-coupled receptor 5 (*LGR5*) and SRY-box transcription factor 9 (*SOX9*) (Fig. 2B), enriched in “stem cell maintenance” pathways (Figs. 2E), indicating a stem cell-like proliferative potential. The proportion of AM-derived cells in Epi3 was significantly increased (Fig. 2F). This finding suggests enhanced epithelial stem cell-like properties and increased proliferative capacity in AM endometrium during the mid-secretory phase, when the endometrium normally completes the transition from proliferation to differentiation. To validate these findings, we examined secretory-phase endometrial tissues. QPCR analysis revealed markedly elevated *LGR5* and *SOX9* expression in AM (Fig. 2G). Immunofluorescence (Fig. 2H and I) confirmed their localization predominantly in epithelial cells, with stronger expression in AM patients. Furthermore, the proliferation marker Ki-67 was significantly upregulated in epithelial cells from AM

patients (Fig. 2J), supporting the presence of aberrant stem cell-like properties and excessive proliferation during the mid-secretory phase. To investigate the roles of *LGR5* and *SOX9* in endometrial epithelial cells, we isolated primary HEECs from mid-secretory endometrium of both healthy controls and patients with AM. In normal HEECs, overexpression of *LGR5* and *SOX9* significantly increased proliferative capacity, as assessed by CCK-8 (Fig. 2K) and EdU assays (Fig. 2L). This was associated with upregulation of the proliferation markers (Ki-67 and PCNA) and downregulation of the secretory marker LIF, as confirmed by qPCR (Fig. 2M), immunofluorescence (Fig. 2N), and western blot analysis (Fig. 2O). Conversely, knockdown of *LGR5* and *SOX9* in AM-derived HEECs led to reduced proliferation, decreased Ki-67 and PCNA expression, and increased LIF levels (Supplementary Fig. 3). These results indicate that *LGR5* and *SOX9* promote epithelial proliferation while suppressing differentiation. Epi4, characterized by *LGALS1* expression (Fig. 2B), contained similar proportions of cells from AM and control samples (Fig. 2F). Galectin-1, encoded by *LGALS1*, is known to contribute to implantation, maternal immune tolerance, placentation, and angiogenesis [32], suggesting a potential role in epithelial–immune–vascular interactions. Collectively, these findings highlight a critical expansion of the *LGR5* *SOX9* Epi3 subcluster in the eutopic endometrium of AM, marked by pronounced stem cell-like properties and hyperproliferation. The failure of these cells to transition into differentiated, receptive epithelium may represent a key mechanism underlying impaired endometrial receptivity in AM.

Aberrant Epi2-to-Epi3 signaling drives stem cell-like properties and hyperproliferation in adenomyosis

To assess alterations in signaling among epithelial subclusters in AM, we applied the CellChat algorithm to systematically analyze ligand–receptor interactions across the four subclusters. The analysis (Fig. 3A) revealed distinct communication patterns between AM and controls. AM epithelial cells exhibited predominant outgoing signals mediated by fibronectin 1 (FN1) [33], semaphorin-5 A (SEMA5A) [34], and junctional adhesion molecule

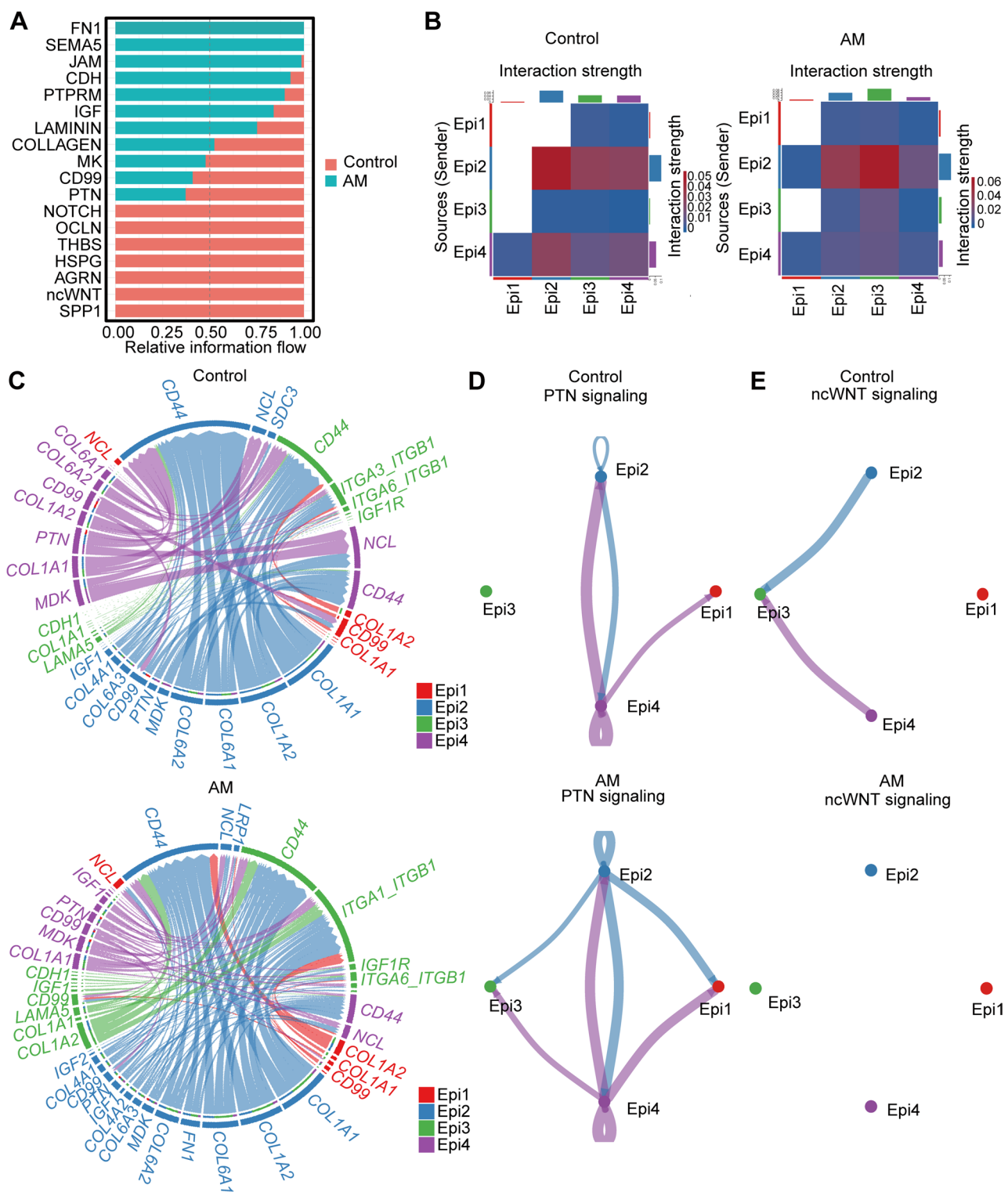


Fig. 3 Rewiring of epithelial subcluster communication networks in adenomyosis (AM). **(A)** Bar graph showing the relative contributions of control and AM groups to individual signaling pathways. **(B)** CellChat-derived heatmap illustrating epithelial (Epi) subcluster interactions in control versus AM samples. **(C)** Circos plot showing NicheNet-predicted ligand–receptor interactions between epithelial subclusters in control and AM groups. The arrow points from the sender to the receiver. **(D–E)** Circle plot depicting the inferred PTN and ncWNT signaling networks between epithelial subclusters in control and AM groups

(JAM) [35], which regulate proliferation, angiogenesis, and tight junction assembly, accompanied by aberrant activation of cell adhesion proteins (CDH, PTPRM) and IGF signaling. These enriched pathways in AM may synergistically drive abnormal epithelial proliferation and enhanced cell–cell adhesion, compromising uterine receptivity and embryo implantation. By contrast, epithelial cells in the control group were enriched for SPP1 [36], non-canonical WNT (ncWNT) [37], and Notch signaling [38], pathways that have been closely associated with epithelial differentiation, receptivity acquisition, and embryo–epithelium interactions. Further analysis of intercellular communication networks (Fig. 3B) revealed substantial remodeling of epithelial interactions in AM patients. Specifically, Epi1 and Epi2 established new signaling connections, while communication from Epi2 to Epi3 was markedly enhanced. Epi2 predominantly transmitted ECM-rich ligands (*FN1*, *COL1A1*, *COL6A1*) and growth factors (*IGF1*, *PTN*) to Epi3, which expressed adhesion receptors (*CD44*, *ITGA1*, *ITGB1*) (Fig. 3C), suggesting reinforced cell–cell adhesion and facilitation of signal transmission. Notably, Pleiotrophin (PTN) signaling from Epi2 to Epi3 was markedly elevated in AM (Fig. 3D). PTN sustains stem cell properties and self-renewal across diverse stem cell systems and promotes proliferation while inhibiting epithelial differentiation [39], suggesting that excessive PTN from Epi2 drives the enhanced stem cell-like properties of the Epi3 subcluster in AM. Conversely, ncWNT signaling from Epi2 to Epi3, present in controls, was absent in AM (Fig. 3E), a pathway essential for epithelial differentiation, functional maturation, progesterone responsiveness, embryo–epithelium interactions, and stromal signaling relevant to decidualization [37, 40]. Its loss may prevent Epi3 cells from completing the proliferative-to-differentiated transition, sustaining stem cell-like properties and hyperproliferation. Together, these findings indicate that AM is associated with profound remodeling of epithelial communication. In particular, the Epi2-to-Epi3 axis shows increased PTN and loss of ncWNT signaling, which may contribute to enhanced stem cell-like properties and impaired differentiation of the Epi3 subcluster.

A SASP-like mature decidual stromal subpopulation is deficient in adenomyosis

As the largest cellular compartment of the endometrium (Fig. 1D), stromal cells play a pivotal role in implantation and decidualization. Despite comparable overall proportions between AM and controls, their predominance, together with accumulating evidence of stromal impairment, motivated us to focus on potential dysfunction within specific subclusters [19]. Accordingly, we focused on stromal (Str) cells and identified four functionally distinct subclusters (Str1–Str4) (Fig. 4A and B). The Str1

subcluster was characterized by high expression of the oxidative stress molecule *SOD2*, complement-related genes *CFD* and *C3*, and the immune-associated molecule *HLA-B*. These features align with established roles of oxidative stress, complement activation, and immune responses in initiating decidualization [6, 41], indicating that Str1 may engage in early immune regulation through interactions with immune cells. In addition, Str1 expressed the calcium-binding protein *S100A4*, a regulator of calcium signaling, cytoskeletal organization, cell migration, and tissue remodeling, which is also upregulated in stromal cells of the normal mid-secretory endometrium [30]. The Str2 subcluster was marked by elevated expression of ECM components (*HSPG2*, *FBLN2*, *CILP*, *ELN*) and *IGF2*, indicating active protein synthesis and secretion and a role in decidual extracellular matrix remodeling. The Str3 subcluster showed enrichment for genes involved in ECM remodeling, including *ECM1*, *MMP11*, and *ADAM12*, as well as the WNT pathway regulator *SFRP4*. ECM remodeling has been recognized as essential for successful implantation and placentation, with metalloproteinases like MMP11 and ADAM12 and their substrates contributing to this process [42]. WNT signaling similarly plays a critical role in stromal decidualization, embryo implantation, and placentation, and SFRP4 expression is decreased in the endometrium of patients with recurrent implantation failure [43]. Notably, the Str4 subcluster co-expressed some metalloproteinases observed in Str3, but specifically exhibited high expression of the TGF β pathway regulator *PMEPA1* [44] and the decidualization-related gene *DIO2* [7, 45]. Single-cell analysis of normal endometrium has highlighted TGF β signaling as a key coordinator of stromal-epithelial interactions [46], implying that Str4 may regulate epithelial function through TGF β signaling. Given that decidualization is a progesterone-driven, progressively enhanced differentiation process, we performed pseudotime analysis on the four stromal subclusters, constructing a developmental trajectory from Str1 to Str4 (Fig. 4C). Functional enrichment analysis (Fig. 4D) revealed a gradual shift in cellular functions along this trajectory: Str1 cells were enriched for RNA processing, protein refolding, and RNA metabolic processes, whereas Str4 cells displayed features typical of decidualization, including cell junction assembly, collagen fibril organization, and extracellular matrix organization. Str4 was further enriched for protein sumoylation and cilium organization, the former linked to progesterone-mediated decidual signaling [47] and the latter essential for decidualization and epithelial–stromal interactions [48]. Integration with pseudotime analysis indicated that decidual-related functions progressively intensified toward Str4, which also expressed high levels of maturation markers including *DIO2*, *PGR*, and *WNT5A*

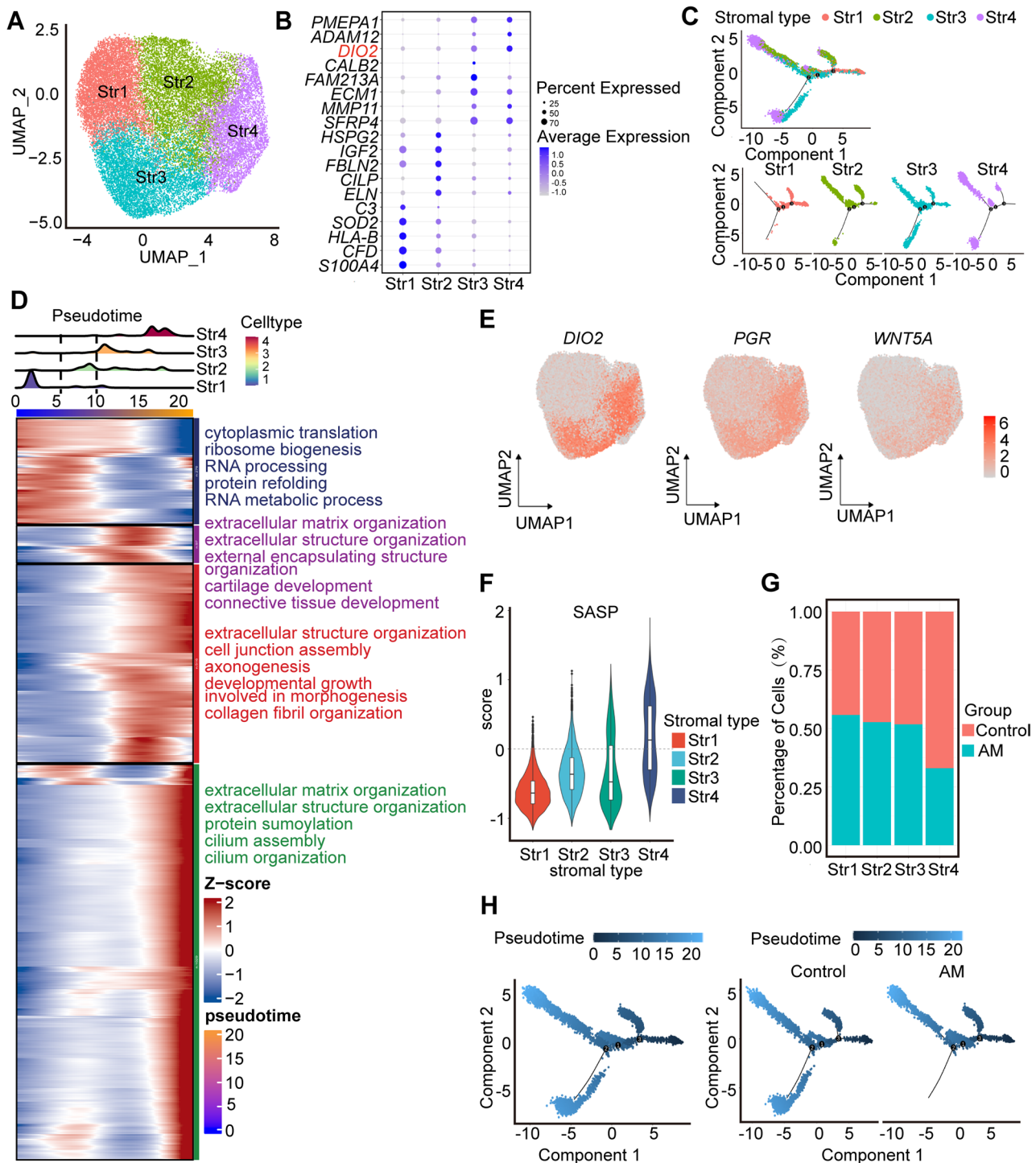


Fig. 4 Identification, functional characterization, and trajectory analysis of stromal cell subclusters in adenomyosis (AM). **(A)** UMAP visualization of stromal subclusters (Str1–Str4). **(B)** Dot plots depicting the average expression of established markers indicated by color. **(C)** Pseudotime trajectory showing the progression of Str1, Str2, Str3 and Str4. **(D)** Gene Ontology (GO) terms significantly enriched across gene clusters with distinct pseudo-temporal patterns. **(E)** The distribution of *DIO2*, *PGR*, and *WNT5A* in stromal cell subclusters by UMAP. **(F)** Violin plot of inflammatory scores across stromal subclusters. **(G)** Box plots of the relative proportions of AM and control cells in each stromal subcluster. **(H)** Separate pseudotime trajectories of stromal subclusters in control and AM groups

(Fig. 4E), identifying this subcluster as the most mature, progesterone-responsive, and epithelium-interactive population. Notably, Str4 specifically expressed *DIO2*, a representative marker of snDCs [7, 45]. SnDCs contribute to decidual responses and modulate the endometrial immune environment through the secretion of SASP factors [7, 8]. SASP components comprise pro-inflammatory cytokines (e.g., IL-6, IL-15), chemokines (e.g., IL-8, CXCL14), growth factor-binding proteins (e.g., IGFBP3), and extracellular matrix-remodeling regulators (e.g., MMPs and their inhibitor TIMP3), whose controlled release supports decidual cell maturation [49]. Consistently, inflammation scoring (Fig. 4F) revealed that Str4 was enriched for inflammatory mediators, further suggesting SASP-like secretory activity and a role in decidual-associated inflammatory regulation. Importantly, in AM patients, the proportion of Str4 cells was significantly reduced (Fig. 4G) and this subcluster was notably absent at the end of the pseudotime trajectory (Fig. 4H).

To validate the single-cell transcriptomic findings, we performed IHC and ELISA on mid-secretory eutopic endometrial tissues from 12 AM patients and 12 controls. *DIO2* was detected in both epithelial and stromal compartments, but stromal expression was markedly reduced in AM (Fig. 5A). Key SASP-related regulators, including CXCL12, IGFBP3, and MMP14 (Fig. 5B) were also significantly diminished in AM. ELISA (Fig. 5C) further revealed a substantial decrease of decidualization markers IGFBP1 and PRL in AM. In vitro decidualization of normal primary HESCs increased senescence-associated β -galactosidase (β -gal) activity (Fig. 5D) and upregulated SASP-related cytokines, including CXCL14, TIMP3, and IL15, at both mRNA (Fig. 5E) and protein levels (Fig. 5F). To investigate the role of *DIO2* in senescence-associated decidualization, normal HESCs were transfected with *DIO2*-targeting siRNA and subsequently treated with MPA and cAMP. Efficient knockdown was confirmed by RT-qPCR (Supplementary Fig. 4A). *DIO2* depletion reduced β -gal staining (Fig. 5G) and suppressed SASP cytokine expression at both mRNA (Fig. 5H) and protein levels (Figs. 5I–K). Morphologically, control cells exhibited typical decidual morphology with cytoskeletal reorganization and a shift from spindle-shaped to rounded forms, reflected by a decreased major-to-minor axis ratio (Fig. 5L and M), which was largely absent in *DIO2*-deficient cells. Conversely, *DIO2* overexpression in AM stromal cells restored β -gal activity and enhanced SASP cytokine expression (Supplementary Fig. 4B–E). Collectively, these data support that Str4 represents a mature decidual population characterized by *DIO2* expression, SASP-like activity, inflammatory responsiveness, and its deficiency in AM may underlie defective decidualization and implantation failure.

We next applied CellChat to systematically map ligand–receptor interactions among the four stromal subclusters. In controls, stromal cells exhibited strong enrichment of classical decidualization-associated pathways, including non-canonical WNT [50], FGF [51], PDGF [52], and IGF signaling [53]. Strikingly, these pathways were entirely absent or markedly attenuated in AM, with IGF signaling showing near-complete loss of IGF1 and IGF1R expression (Fig. 6A, B and Supplementary Fig. 5). In the normal endometrium, Str4 served as a central communication hub, integrating inputs from Str1, Str2, and Str3. By contrast, in AM, this network was severely disrupted: input from Str2 to Str4 was lost, and connections from Str1 and Str3 to Str4 were markedly weakened (Fig. 6C). The complete loss of ncWNT signaling among AM stromal subclusters (Fig. 6D) further underscores a breakdown in the normal differentiation trajectory, impairing the transition from early stromal states toward the mature, decidual Str4 phenotype.

Disrupted bidirectional epithelial–stromal crosstalk impairs endometrial receptivity in adenomyosis

Endometrial receptivity relies on finely tuned bidirectional communication between epithelial and stromal cells. To dissect these interactions during the mid-secretory phase, we first applied NicheNet to examine epithelial-to-stromal signaling (Fig. 7A). GO analysis of the top 20 epithelial-derived ligands (Fig. 7B) revealed enrichment for cell junction organization, cell–cell adhesion, and secretory processes—mechanisms critical for epithelial membrane remodeling and acquisition of receptivity [54]. The predicted stromal targets of these ligands were predominantly involved in angiogenesis, tissue remodeling, cell migration, and cytokine responses (Fig. 7C), suggesting that epithelial signals orchestrate stromal decidualization and create a favorable microenvironment for implantation. Among epithelial-derived signals, IGF2 showed the highest interaction frequency with stromal targets (Fig. 7A), including key decidualization regulators (*DIO2*, *IGFBP5*, *PAPP*), SASP-associated factors (*IL32*, *CXCL12*), and extracellular matrix components (*COL1A1*, *VCAN*, *LAMA4*, *COL5A1*). In AM patients, IGF2 signaling was significantly reduced (Fig. 7D), indicating impaired epithelial–stromal communication. Another prominently downregulated signal in AM was DKK1, a canonical WNT antagonist that targets the transcription factor LEF1 in stromal cells. In AM, DKK1 expression is markedly reduced (Fig. 7E), accompanied by decreased stromal LEF1 [19], indicating a disruption of WNT-mediated epithelial–stromal crosstalk that compromises decidualization. To explore the potential role of PTN/ncWNT signaling in epithelial-stromal crosstalk, we focused on DKK1, a ncWNT pathway regulator that was prominently recruited in our intercellular

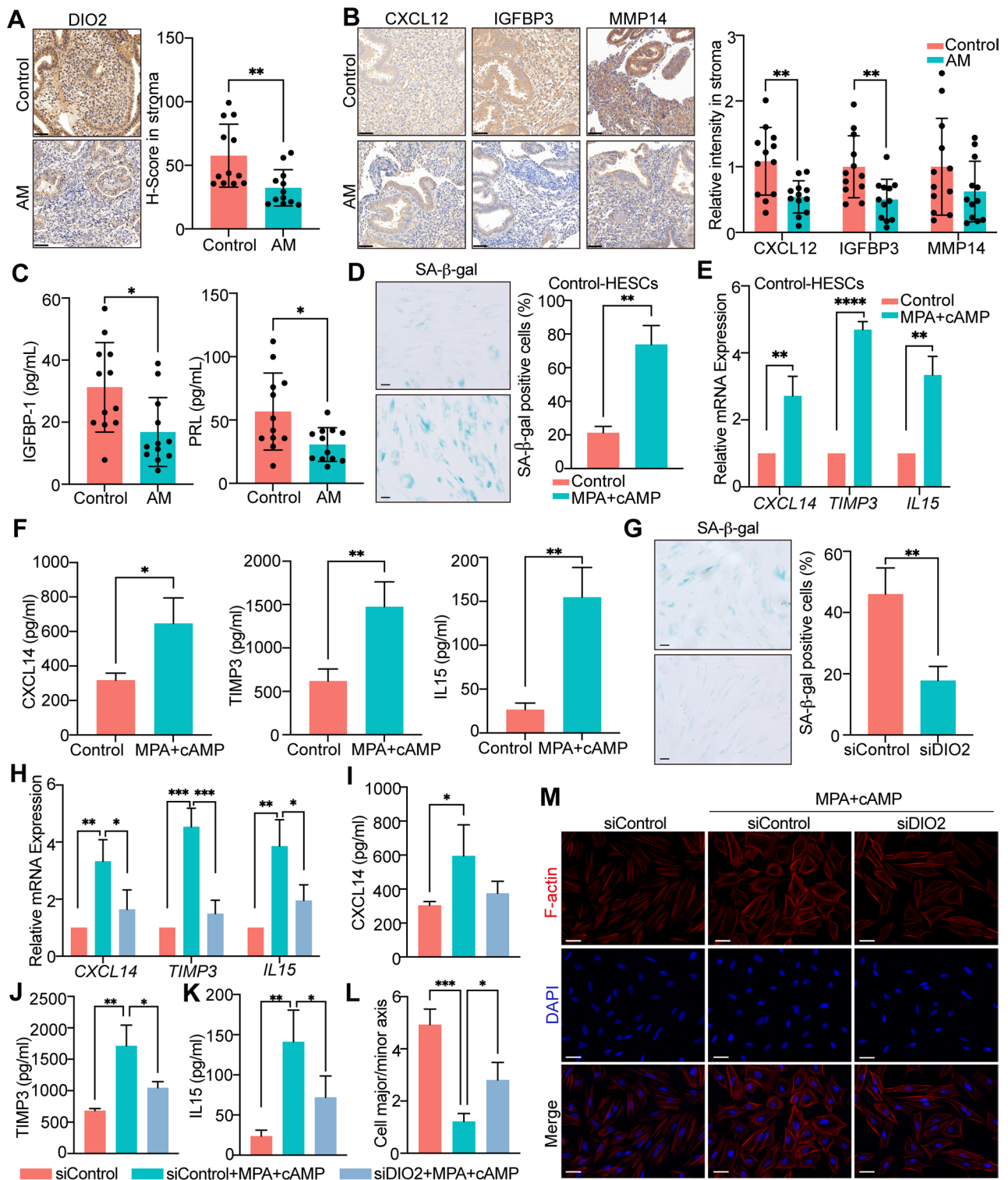


Fig. 5 DIO2 Str4 stromal cells exhibit SASP-like activity and drive decidualization. **(A, B)** Representative immunohistochemistry (IHC) images of DIO2, CXCL12, IGFBP3, and MMP14 in mid-secretory eutopic endometrial tissues from controls and adenomyosis (AM) patients ($n = 12$). Scale bars, 50 μ m. **(C)** ELISA quantification of decidualization markers IGFBP1 and PRL in control and AM tissues ($n = 12$). **(D)** Senescence-associated β -galactosidase (SA- β -gal) staining in control HESCs undergoing in vitro decidualization. **(E)** RT-qPCR analysis of SASP-related cytokines CXCL14, TIMP3, and IL15 in decidualized control HESCs ($n = 3$). **(F)** ELISA measurement of secreted SASP cytokines in culture supernatant ($n = 3$). **(G–K)** Effects of DIO2 knockdown on β -gal activity **(G)**, SASP cytokine mRNA expression **(H)**, and secreted protein levels **(I–K)** in control HESCs ($n = 3$). **(L, M)** F-actin filaments staining in control HESCs following DIO2 knockdown during in vitro decidualization, visualized with Alexa Fluor 555–conjugated phalloidin. Scale bar = 50 μ m, $n = 3$. Data are presented as the mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

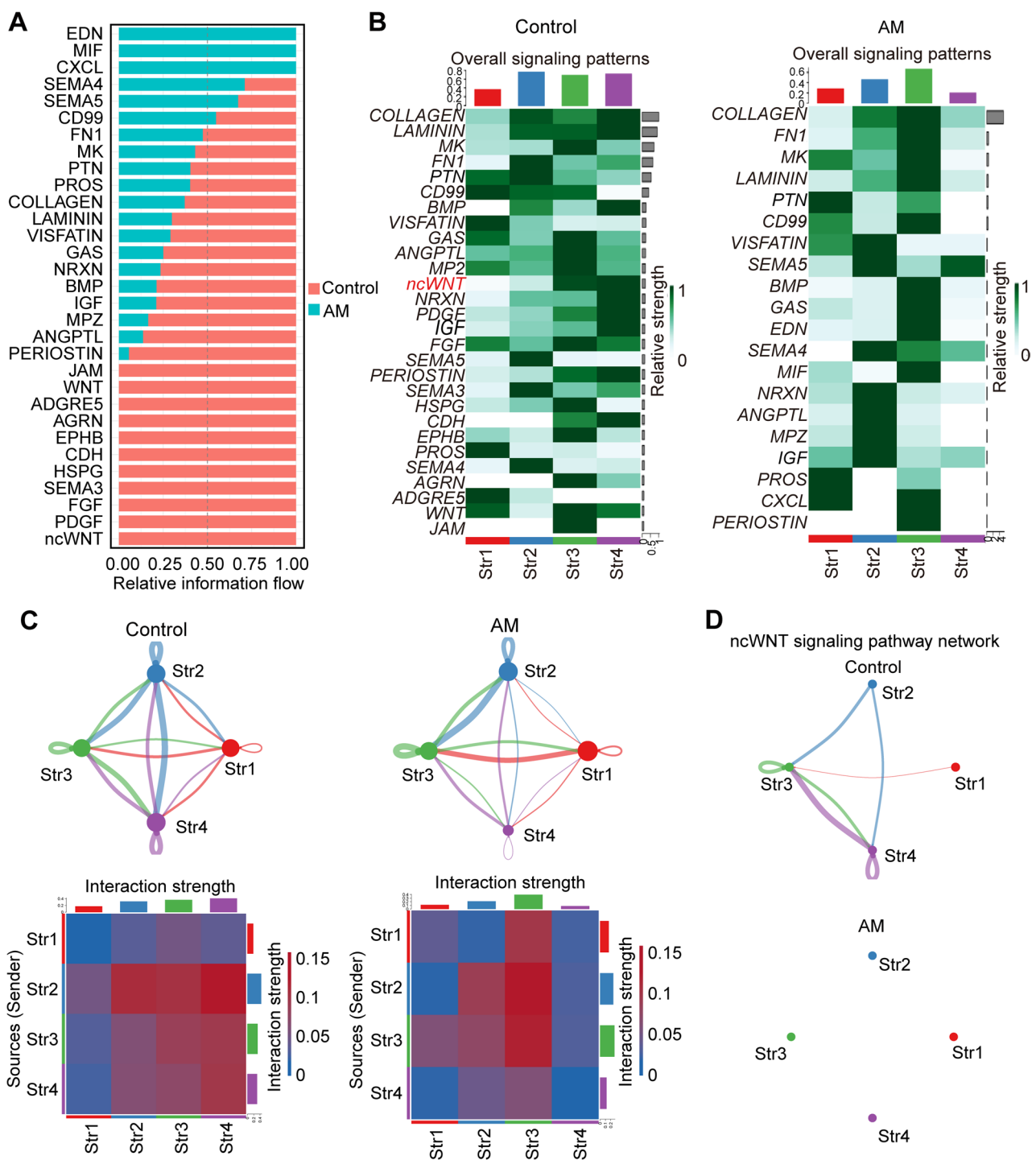


Fig. 6 Altered communication networks among stromal subclusters in adenomyosis (AM). **(A)** Bar graph showing the relative contributions of control and AM groups to individual signaling pathways across stromal subclusters. **(B)** Heatmap of stromal subcluster (Str) signaling patterns in control and AM inferred by CellChat. **(C)** Circle plot (upper) and heatmap (lower) illustrating inter-subcluster interactions in control vs. AM. **(D)** Circle plot of inferred ncWNT signaling networks among stromal subclusters in control and AM

signaling analysis (Supplementary Fig. 6). Treatment of normal primary epithelial cells with DKK1 or a DKK1 inhibitor, followed by collection of conditioned media to treat stromal cells undergoing decidualization, showed that DKK1 promoted IGFBP1 and PRL expression while DKK1 inhibition had the opposite effect, suggesting that epithelial-stromal interactions are at least partly ncWNT-dependent. At the subcluster level (Fig. 7F),

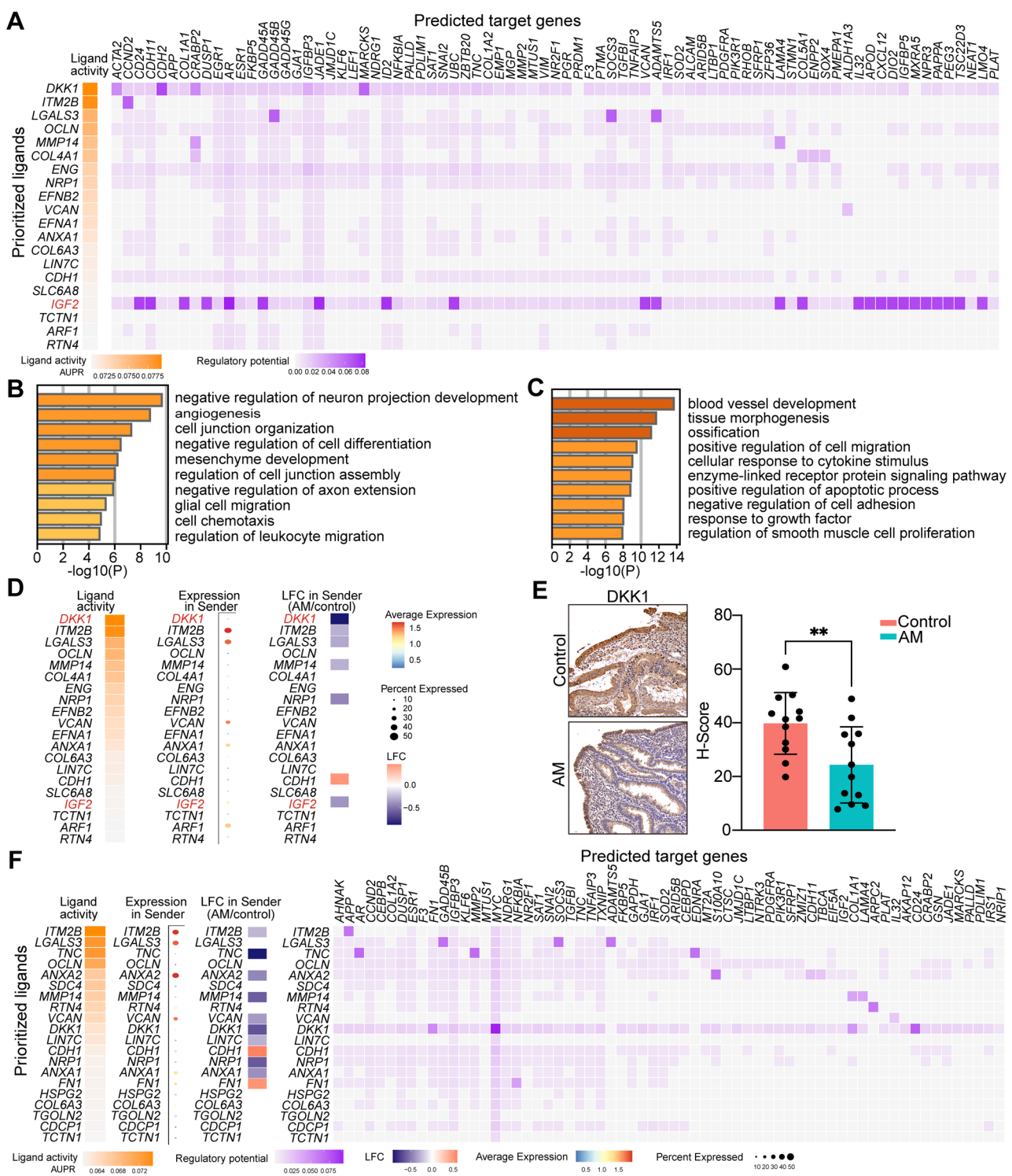


Fig. 7 Aberrant epithelial-to-stromal communication in mid-secretory endometrium of adenomyosis (AM). **(A)** A Heatmaps (NicheNet) of ligand activity of top-ranking ligands expressed by epithelial cells (left) and their regulatory potential on predicted target genes expressed by stromal cells (right). **(B)** Gene Ontology (GO) enrichment analyses of epithelial ligands. **(C)** GO enrichment of predicted stromal targets. **(D)** NicheNet heatmaps showing ligand activity (left) and expression (middle) of top epithelial-derived ligands, with log fold change (LFC, right) indicating differential expression between AM and controls. **(E)** Representative immunohistochemistry images of DKK1 in mid-secretory eutopic endometrium from control and AM patients. Scale bars, 50 μ m. **(F)** NicheNet heatmaps displaying ligand activity of Epi3 epithelial cells (left) and their regulatory influence on Str4 stromal targets (right)

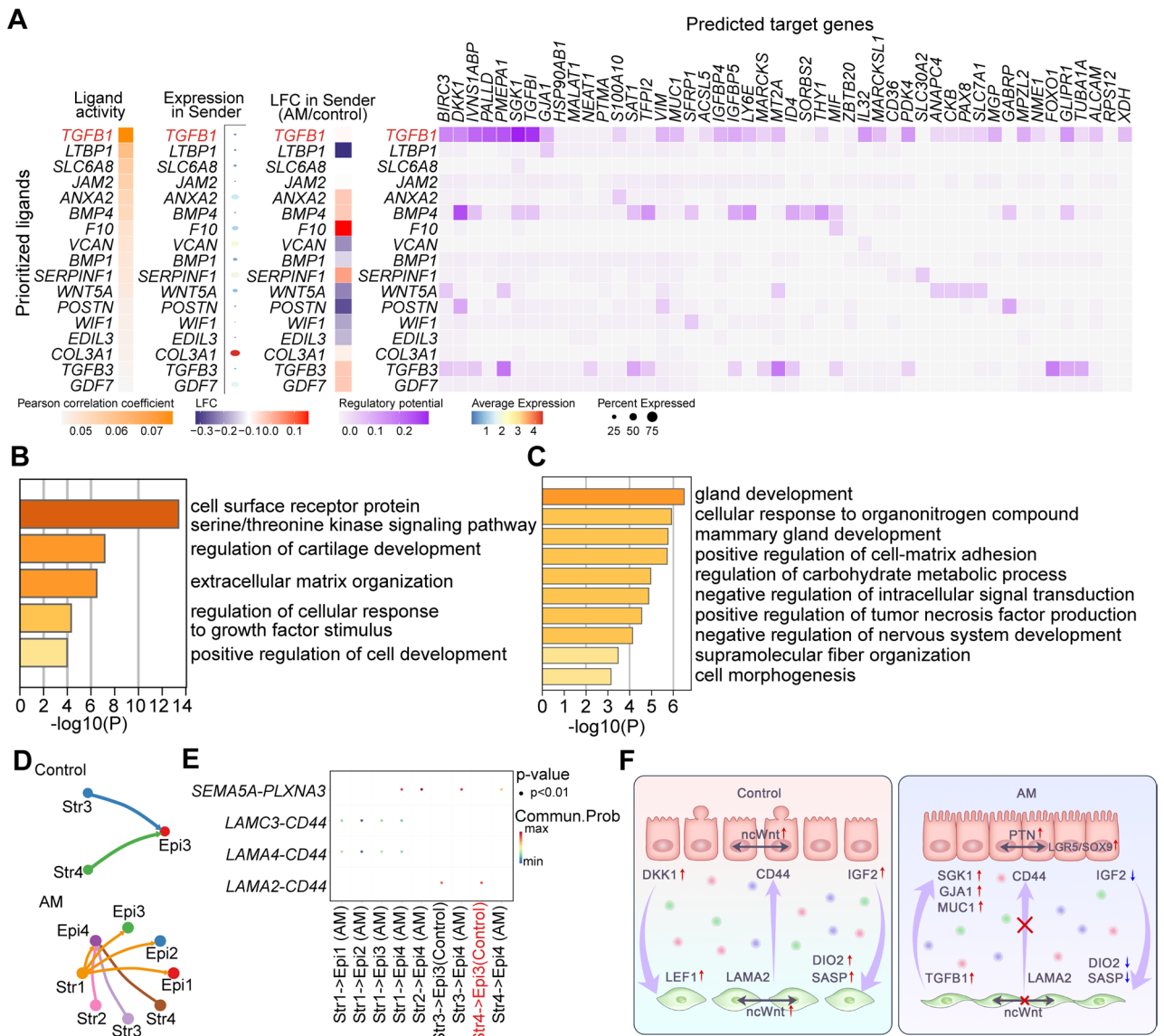


Fig. 8 Alterations in stromal-to-epithelial signaling in adenomyosis (AM). **(A)** NicheNet heatmaps showing activity of top stromal-derived ligands (left) and their predicted regulatory impact on epithelial targets (right). **(B)** Gene Ontology (GO) enrichment of stromal ligands. **(C)** GO enrichment of epithelial targets. **(D)** Circle plot depicting inter-subcluster inter-subcluster signaling between stromal (Str) and epithelial (Epi) cells in controls versus AM. **(E)** Dot plots illustrating representative ligand–receptor interactions across these subclusters in both groups. **(F)** Schematic summary of epithelial–stromal cross-talk alterations in adenomyosis

Epi3 epithelial cells transmit multiple signals—including *ITM2B*, *LGALS3*, *DKK1*, *VCAN*, *MMP14*, and *OCN*—to the mature decidual stromal subcluster Str4. The predicted targets of these signals are enriched in pathways related to inflammatory responses (*IL32*, *MMP2*), hormone regulation (*ESR1*), stress response (*NR4A1*, *GADD45B*), and decidualization (*IGFBP3*, *FKBP5* [55]), highlighting their critical role in Str4 maturation. In AM, these epithelial–stromal signals are variably disrupted, impairing stromal maturation.

Conversely, stromal-to-epithelial signaling analysis identified 17 candidate ligands (Fig. 8A), enriched in

ECM remodeling, cell development, and serine/tryptophan metabolism (Fig. 8B), all critical for stromal decidualization. Their epithelial targets were enriched in glandular differentiation, tissue morphogenesis, and cell–matrix adhesion pathways (Fig. 8C), highlighting a reciprocal regulatory axis. Among these, *TGFB1* emerged as the most active signal, targeting polarity regulator *SGK1*, gap junction protein *GJA1*, and mucin *MUC1*, all intimately linked to epithelial remodeling. Suppression of *MUC1* and *GJA1* is required for receptivity [56, 57], and persistent aberrant *SGK1* expression can impair implantation [58]. Notably, *TGFB1* signaling was paradoxically

enhanced in AM (Fig. 8A), potentially disturbing epithelial polarity. Subcluster interaction analysis revealed a complete loss of signaling from Str4 to Epi3 in AM, particularly the LAMA2–CD44 ligand–receptor pair (Fig. 8D and E). LAMA2, a key ECM protein, transmits intercellular cues via CD44 [59] and has been shown to restrain excessive proliferation while promoting differentiation in neural stem cells [60]. CD44 is critical for epithelial polarity remodeling and embryo adhesion during the mid-secretory phase, and its reduced expression is associated with impaired decidualization [61]. Disruption of this pathway likely diminishes differentiation signals to Epi3 epithelial stem cells, hindering the establishment of the non-polarized state required for endometrial receptivity. In summary, epithelial–stromal interactions during mid-secretory phase are profoundly perturbed in AM, with altered ligand–receptor signaling that promotes epithelial stem cell-like properties while inhibiting epithelial polarity remodeling and stromal decidualization (Fig. 8F).

Furthermore, we analyzed communication between immune cells and other endometrial cell types. Cell–cell communication analysis revealed no significant difference in overall signaling between AM and control groups (Supplementary Fig. 7A). Stratification into lymphoid populations (including uNK cells) and macrophages similarly showed no change in global communication intensity (Supplementary Fig. 7B). Immune–stromal interactions were consistently stronger than immuneepithelial interactions across both cohorts. In AM, the dominant immune–stromal signaling axis was *IFNG/IFNGR*, whereas in control endometrium, *GZMA/F2R* and *GZMA/PARD3* interactions prevailed (Supplementary Fig. 7C). These data indicate that while the overall magnitude of immune–stromal communication is comparable, the qualitative composition differs between AM and control endometrium.

Discussion

Embryo implantation is a highly coordinated process that requires the endometrium to acquire an optimal receptive state during the window of implantation. In recent years, increasing attention has been given to receptivity defects in the eutopic endometrium of patients with AM [62]. Proposed mechanisms include aberrant hormonal signaling [63], immune dysregulation [64], and abnormal angiogenesis [65], yet the precise cellular and molecular landscape during this critical period remains poorly defined. Unlike endometriosis, where lesions are ectopic, AM involves invasion of endometrial glands and stroma into the myometrium, causing junctional zone disruption, intramural remodeling, and localized inflammation [66]. Our high-resolution scRNA-seq analysis of mid-secretory eutopic endometrium reveals AM-specific

alterations: aberrant expansion of epithelial subclusters with stem cell-like properties and proliferative properties, impaired differentiation of stromal cells toward a mature decidual phenotype with SASP-like features, and disrupted epithelial–stromal crosstalk. These alterations may synergistically impair endometrial receptivity and contribute to implantation failure in AM.

The endometrial epithelium is the first maternal cell type encountered by the blastocyst during adhesion and implantation, playing a central role in establishing the maternal–fetal interface [67]. Single-cell profiling revealed that, while the overall cellular composition was similar between groups, AM samples exhibited a pronounced expansion of epithelial cells during the mid-secretory phase. This finding aligns with previous reports of glandular hyperplasia in the basal layer and ectopic lesions of AM [27, 68], suggesting that epithelial abnormalities may be a shared feature of both eutopic and ectopic endometrium. We identified three epithelial subclusters (Epi1–Epi3) with distinct transcriptional signatures that were significantly expanded in AM. Epi1 cells, marked by metallothionein family members associated with the proliferative-to-secretory transition [30, 69], normally diminish as the cycle advances. Their persistence in AM during the mid-secretory phase suggests a delayed transition from proliferation to differentiation, which may result in a displaced or postponed implantation window, consistent with clinical observations of delayed implantation in AM [70]. The Epi2 subcluster in AM exhibited aberrantly enhanced tight junction activity and persistent estrogen signaling, deviating from the progesterone-driven remodeling essential for receptivity. Normally, receptivity establishment involves downregulation of estrogen signaling, cessation of epithelial proliferation, and transition to a differentiated phenotype, accompanied by cytoskeletal rearrangement, loosening of tight junctions, and loss of apical microvilli [5]. In AM, dysregulated steroid hormone signaling—characterized by local estrogen dominance and progesterone resistance—likely contributes to these epithelial abnormalities [21]. Elevated estradiol levels and altered receptor expression (upregulated ER β , downregulated PR-B) promote epithelial proliferation and inflammation while impairing stromal decidualization, reducing expression of implantation-associated markers such as HOXA10 and LIF [16]. Persistent tight junction activity in Epi2 may hinder epithelial remodeling, delay receptivity onset, and interfere with blastocyst–endometrium interactions. Previous studies have reported sustained expression of tight junction proteins such as Claudin-10 [71] and downregulation of receptivity markers such as LIF and HOXA10 [19] in AM mid-secretory endometrium, supporting this hypothesis. Collectively, the persistence of proliferative Epi1 and the abnormal junctional reinforcement of Epi2

delineate distinct epithelial dysfunctions that converge on receptivity impairment in AM.

Most notably, the Epi3 subcluster highly expressed stem cell markers LGR5 and SOX9, enriched in stem cell maintenance pathways, and exhibited strong proliferative potential. Importantly, LGR5 cells have been shown to play a key role in uterine gland development [72], whereas SOX9 epithelial cells peak during the proliferative phase and decline during differentiation, establishing a precise window for embryo implantation [46]. Their temporally coordinated expression is thus essential for maintaining a normal endometrial cycle. Importantly, AM is increasingly recognized as a disorder driven by repeated cycles of tissue injury and repair at the endometrial–myometrial interface, accompanied by chronic inflammation and progressive fibrotic remodeling [1, 27]. In such contexts, epithelial progenitor-associated programs are often activated as part of a regenerative response to sustained injury signals. Consistent with this paradigm, enrichment of LGR5/SOX9 epithelial cells has been observed not only in AM lesions [23] but also in peritoneal endometriotic lesions [73], suggesting that chronic inflammatory microenvironments engage stem-like epithelial states across disease compartments. In our study, the marked expansion of Epi3 in the eutopic endometrium of AM patients, together with IHC confirming upregulation of LGR5, SOX9, and the proliferation marker Ki-67, suggests that chronic inflammatory remodeling in AM extends beyond ectopic lesions and actively reshapes epithelial cell states *in situ*. This expanded epithelial subset maintains stem cell-like properties and proliferative activity while failing to differentiate during the secretory phase—a likely contributor to impaired receptivity. These findings raise the possibility that sustained activation of progenitor-associated epithelial programs in the context of chronic injury and inflammation may be linked to epithelial dysregulation and reduced endometrial receptivity in AM. Further cell–cell communication analysis revealed a profound rewiring of ligand–receptor interactions in AM epithelium, with the most pronounced changes observed in signaling from Epi2 to Epi3. In AM, PTN signaling from Epi2 was markedly enhanced, whereas ncWNT pathway activity was nearly abolished. PTN, a cytokine closely associated with stem cell properties maintenance, is known to inhibit epithelial differentiation while promoting proliferation [36]. Consistently, single-cell studies have shown reduced PTN signaling in thin endometrium [74] but elevated levels in adenomyotic lesions [23]. Mechanistically, PTN has been shown to enhance β -catenin stabilization and nuclear accumulation, thereby promoting β -catenin/TCF-dependent transcription and proliferative programs [75]. Normally, canonical WNT is downregulated during the implantation window to allow epithelial depolarization,

junctional loosening, and morphological remodeling, whereas persistent activation can impair receptivity [76]. By contrast, ncWNT signaling is critical for establishing receptivity: WNT5a and WNT11 activate the ncWNT/PCP pathway to remodel the epithelial cytoskeleton [50], while WNT7a trigger ncWNT/ Ca^{2+} signaling to enhance embryo–epithelium adhesion [77]. In addition, multiple studies have demonstrated that dominant activation of canonical WNT/ β -catenin signaling can antagonize ncWNT/PCP and ncWNT/ Ca^{2+} outputs, not by directly inhibiting ncWNT ligands, but by shifting pathway balance toward β -catenin-dependent cell fate and proliferation programs [78, 79]. Taken together, in AM, enhanced PTN signaling from Epi2 to Epi3 likely drives persistent proliferation of the Epi3 subcluster via canonical Wnt activation, while loss of ncWNT signaling disrupts differentiation cues. This dual alteration promotes expansion of the LGR5/SOX9 Epi3 population, sustaining stem-like activity and hyperproliferation, which in turn interferes with normal epithelial differentiation and remodeling, ultimately impairing endometrial receptivity and providing a mechanistic explanation for AM-associated infertility.

Proper decidualization of stromal cells is essential for successful embryo implantation and pregnancy maintenance [10]. While the overall stromal cell proportion in AM endometrium is largely unchanged, subcluster and pseudotime analyses indicate substantial impairment in the decidualization process. Notably, Str4 represents a mature decidual subpopulation marked by high DIO2 expression and SASP enrichment, characteristic of snDCs [7]. Decidualization is initiated by conserved acute stress responses, including rapid release of pro-inflammatory factors and transient ROS elevation [80, 81], followed by morphological transition from fibroblast-like to secretory decidual cells. Concomitantly, snDCs are induced, transiently expressing senescence markers such as p16^{INK4}, and producing SASP to amplify early inflammatory responses, promote synchronous stromal differentiation, and activate receptivity-related genes [7, 8]. These acute senescent cells are then cleared by uNK cells, maintaining tissue homeostasis and preventing prolonged damage [82]. Although snDCs contribute to physiological decidual remodeling, excessive accumulation—particularly of DIO2⁺high cells—has been linked to recurrent pregnancy loss [45], suggesting a dual role in regulating the decidual response. Physiological, transient senescence and SASP support implantation by generating a controlled pro-inflammatory milieu, recruiting immune regulators, and facilitating dynamic tissue remodeling [83]. In contrast, excessive or unresolved accumulation of senescent cells can produce sustained SASP, creating a chronic inflammatory microenvironment, impairing stromal hormone responsiveness, and diminishing decidual differentiation

capacity, which collectively compromise endometrial receptivity [84]. In AM, the deficiency of this acute senescent subpopulation likely undermines the completeness and functionality of decidualization, compromising endometrial receptivity.

Endometrial receptivity is a prerequisite for successful embryo implantation, relying on finely tuned, dynamic crosstalk between epithelial and stromal cells. In the normal cycle, epithelial-derived signals promote stromal decidualization, which in turn regulates epithelial function to establish a supportive implantation micro-environment [85]. In AM, this interdependent communication network is disrupted. We observed a pronounced loss of ncWNT signaling in both epithelial and stromal compartments, accompanied by significant downregulation of epithelial DKK1, a progesterone-regulated WNT inhibitor that facilitates stromal decidualization [86]. Aberrant WNT/SFRP signaling in AM lesions has also been implicated in disease pathogenesis [23, 68], suggesting that WNT dysregulation may directly impair endometrial receptivity. Our epithelial–stromal interaction experiments using DKK1 indicate that these interactions are at least partly ncWNT-dependent. Although preliminary, these findings support a model in which ncWNT signaling coordinates communication between epithelial and stromal compartments. Future studies employing endometrial organoids with targeted modulation of PTN or key ncWNT components in co-culture with stromal cells will be essential to establish causality and elucidate the underlying mechanisms of epithelial–stromal crosstalk.

NicheNet analysis further revealed that mid-secretory epithelial ligands predominantly influence cell adhesion and junction remodeling—prerequisites for establishing a non-polarized state—while downstream stromal targets are associated with angiogenesis, tissue remodeling, migration, and cytokine responses, underscoring the role of epithelial cues in activating stromal decidual programs. Notably, IGF2 emerged as a key epithelial signal, regulating decidual factors, SASP molecules, and ECM components, and directly promoting stromal decidualization [87]. IGF2 also enhances epithelial lysosomal activity via STAT3, facilitating degradation of “non-receptive” markers such as CLDN1 and MUC1, thereby supporting the acquisition of a receptive phenotype [13]. Its downregulation in AM indicates a loss of positive epithelial-to-stromal regulatory input. Importantly, stromal IGF signaling is also essential for decidualization: single-cell study has identified an IGF1–IGF1R axis within stromal compartments that coordinates inter-stromal differentiation, and disruption of this pathway is associated with impaired receptivity and recurrent implantation failure [53]. Consistently, *in vitro* decidualization of human endometrial stromal cells is accompanied by

upregulation of IGF signaling components, including IGF1R [88]. In this context, the marked attenuation of stromal IGF signaling observed in AM suggests that loss of reciprocal stromal IGF input may further exacerbate epithelial–stromal communication defects. Conversely, stromal-to-epithelial signaling also contributes to receptivity. In our study, decidual stromal cells were found to express ligands such as TGFB1 to modulate epithelial polarity, differentiation, and glandular function. TGFB1 target genes included SGK1, GJA1, and MUC1, which regulate membrane polarity; their overactivation can impair establishment of a non-polarized epithelial state, leading to implantation failure [57, 58]. Single-cell studies of normal endometrium similarly support a key role for TGF β signaling in coordinating stromal-epithelial functions [46]. Emerging evidence indicates that the eutopic endometrium in adenomyosis and endometriosis is not biologically normal, exhibiting altered inflammatory gene expression and extracellular matrix remodeling, consistent with chronic inflammation and fibrotic changes [66]. TGFB1 is a central driver of fibrogenesis in endometrial tissues, promoting Smad2/3-dependent extracellular matrix deposition, myofibroblast differentiation, and fibrotic remodeling [89, 90]. In this context, the enhanced stromal-to-epithelial TGFB1 signaling observed in AM may reflect ongoing fibrotic remodeling, which can further disrupt epithelial polarity and stromal–epithelial crosstalk. In addition, TGFB1 is associated with fibrosis-type SASP from chronically senescent stromal cells [91], which suppress decidual factor secretion such as PRL and IGFBP1, further compromising receptivity [92]. In AM, enhanced TGFB1 signaling may thus drive a “pathological decidualization” state that disrupts epithelial receptivity. Regarding aberrant epithelial proliferation, current evidence suggests a bidirectional relationship with stromal defects rather than a unidirectional cause–effect sequence. In AM, insufficient progesterone signaling and persistent estrogen exposure can directly drive excessive epithelial proliferation and delay differentiation, increasing expression of proliferative and progenitor-associated transcripts [93]. Simultaneously, defective stromal decidualization due to progesterone resistance fails to provide homeostatic paracrine signals necessary to constrain epithelial growth [85]. Consistently, our single-cell analysis reveals that in AM, aberrantly proliferative and tightly connected epithelial cells reduce positive signals such as IGF2, thereby limiting stromal decidualization, while incompletely decidualized stromal cells produce abnormal SASP factors, including TGFB1, which impair epithelial polarity and receptive phenotype acquisition. This dual-feedback disruption breaks the epithelial–stromal network, providing a mechanistic basis for implantation failure and infertility in AM.

In the analysis of immune cell interactions with other endometrial cell types, although overall communication strength between immune cells and other compartments did not differ between AM and control groups, the qualitative composition of signaling was markedly altered. Immune–stromal interactions predominated over immune–epithelial interactions in both cohorts, consistent with prior single-cell studies [26]. In AM, IFNG/IFNGR1 + IFNGR2 dominated the immune–stromal signaling landscape, reflecting a shift toward proinflammatory, anti-decidualization pathways. Aberrant IFN signaling has been linked to impaired stromal differentiation and adverse reproductive outcomes, including early pregnancy loss and trophoblast dysfunction [94, 95]. In contrast, control endometrium exhibited granzyme A–associated interactions (GZMA/F2R, GZMA/PARD3), which support regulated stromal turnover and immune homeostasis [96]. These findings suggest that, while the magnitude of immune–stromal communication is comparable, AM is characterized by a skewed signaling profile that may compromise stromal function and endometrial receptivity.

This study had certain limitations. First, the sample sizes of both the scRNA-seq cohort ($n=3$ per group) and the validation cohort ($n=12$ per group) were relatively limited, which may not fully capture the biological heterogeneity of adenomyosis. Although clear and consistent trends were observed, larger cohorts and multi-center datasets will be required to improve statistical power, enhance generalizability, and validate our findings. Future studies integrating expanded sample sizes and emerging scRNA-seq datasets specifically covering the implantation window will be particularly valuable. Second, from a clinical perspective, patients with adenomyosis required a greater number of embryo transfers and transferred embryos yet achieved lower live birth rates, supporting the concept that implantation failure in adenomyosis is primarily driven by endometrial dysfunction rather than embryo quality. The elevated epithelial expression of LGR5 and SOX9 identified in adenomyosis may reflect a maladaptive endometrial state characterized by excessive proliferation and impaired receptivity. However, given the limited sample size, the present study does not establish robust correlations between LGR5/SOX9 expression and implantation or live birth outcomes. Well-powered retrospective and prospective clinical studies will therefore be necessary to assess their predictive value and clinical relevance. Finally, all functional experiments were performed in primary cell cultures, which, while informative, cannot fully capture the complex architecture, immune cell interactions (e.g., uNK cells, macrophages), or hormonal influences (such as progesterone resistance or estrogen dominance) present in the endometrium. These factors may contribute

to aberrant epithelial proliferation and impaired stromal decidualization in AM. Future studies using endometrial organoids co-cultured with stromal and immune cells under controlled hormonal conditions will be critical to better model these interactions and validate the functional significance of our findings.

Conclusion

In conclusion, this study provides a single-cell-level characterization of mid-secretory eutopic endometrium in AM from the perspective of receptivity defects. We identified an expanded epithelial subpopulation characterized by high expression of stem cell markers LGR5 and SOX9, accompanied by sustained proliferation; a loss of DIO2⁺ SASP-positive decidual stromal cells; and a deeply rewired epithelial–stromal communication network marked by abnormal activation of PTN and TGFB1 signaling and loss of ncWNT and IGF2 signaling. Together, these findings establish a mechanistic link between aberrant epithelial proliferation, stromal decidualization failure, and disrupted intercellular communication, providing concrete molecular clues to the implantation defects observed in AM.

Abbreviations

AM	Adenomyosis
scRNA-seq	Single-cell RNA sequencing
IVF/ICSI	In vitro fertilization/intracytoplasmic sperm injection
WOI	Window of implantation
ESCs	Endometrial stromal cells
cAMP	Cyclic AMP
FOXO1	Forkhead box O1
snDCs	Senescent decidual cells
SASP	Senescence-associated secretory phenotype
FGFs	Fibroblast growth factors
IGF-1	Insulin-like growth factor-1
LIF	Leukemia inhibitory factor
HOXA10	Homeobox A10
TVUS	Transvaginal ultrasonography
MRI	Magnetic resonance imaging
BMI	Body mass index
PCA	Principal component analysis
IGFBP1	Insulin like growth factor binding protein 1
PRL	Prolactin
RT-qPCR	Quantitative real-time reverse transcription polymerase chain reaction
IF	Immunofluorescence
IHC	Immunohistochemistry
ELISA	Enzyme-linked immunosorbent assay
Epi	Epithelial
Str	Stromal

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Not applicable.

Authors' contributions

B. X. and A. Z. initiated and supervised the project. G. T. initiated and analyzed the data. Y. Z. and C. H. performed the experimental work, analyzed the data and prepared the original draft manuscript. M. X., J. H. and Y. H. collected the clinical samples and data. W. Z. collected the clinical samples and analyzed the data. X. Z. revised and polished the manuscript. H. Z. and Y. L. contributed to experimental work. D. Z. revised the manuscript. All authors critically read and commented on the manuscript and approved the final version for submission.

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Data availability

All data is available in the main text or the supplementary materials. The raw sequence data from patients with AM reported in this paper have been deposited in the Genome Sequence Archiv [97] in National Genomics Data Center [98], China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: HRA012445) that are publicly accessible at <https://ngdc.cncb.ac.cn/gsa-human>. The raw data for the control single-cell RNA-seq samples are available from the NCBI Gene Expression Omnibus (GEO) under accession number GSE183837.

Declarations

Ethics approval and consent to participate

All procedures concerning human samples were approved by the Institutional Ethics Committee of Ruijin Hospital, School of Medicine, Shanghai Jiao Tong University (no. KY2020-3) and followed the Declaration of Helsinki for human research. Written informed consent was obtained from all participants.

Consent for publication

Written informed consent has been obtained from each patient to publish this paper.

Competing interests

The authors declared that they have no competing interests.

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