

Patient-derived human endometrial organoids recapitulate epithelial polarity and hormone-responsive features associated with endometrial receptivity

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Abstract

Background

Human endometrial organoids (hEOs) provide a three-dimensional model for studying endometrial physiology and cyclic remodeling. However, their transcriptional stability during long-term expansion, inter-individual variability, and responses to physiological hormonal stimulation remain insufficiently characterized. This study established patient-derived hEO lines and assessed their long-term phenotypic and transcriptional stability and hormone-responsive transcriptional programs.

Methods

Endometrial organoids were derived from biopsy samples obtained from 19 individuals. Glandular architecture, secretory activity, and epithelial polarity were evaluated using hematoxylin and eosin staining, periodic acid–Schiff staining, and immunofluorescence. Bulk RNA sequencing was performed to assess inter-individual and passage-associated transcriptional variation. To model proliferative- and secretory-phase conditions, hEOs were treated with estradiol (E2) alone or with estradiol, progesterone, and cyclic adenosine monophosphate (EPC), respectively. Morphological changes, receptivity-associated markers, and genome-wide transcriptional responses were subsequently evaluated.

Results

Eighteen stable hEO lines were established, corresponding to a derivation efficiency of 94.7%. The organoids were maintained for more than 4 months and 12 passages while retaining gland-like luminal architecture, epithelial polarity, secretory activity, and expression of E-cadherin, epithelial cell adhesion molecule, cytokeratin 5, mucin 1, and laminin β 1. Transcriptomic profiles were highly reproducible between biological replicates, and 75.4% of detected genes remained stable across passages. Passage-associated changes predominantly involved inflammatory and immune-related pathways, whereas patient-specific transcriptional features persisted during expansion. Both hormonal regimens promoted organoid growth and increased the expression of the receptivity-associated genes *PAEP*, *SPP1*, and *HSD17B2*. E2 and EPC elicited overlapping but distinct transcriptional responses. *SERPINA3*, *S100P*, *TFPI2*, and *KCNG1* were upregulated under both conditions, whereas *ARL4D*, *ENPP3*, and *COL1A2* were selectively upregulated after EPC treatment. E2 increased progesterone receptor expression, and EPC induced broader transcriptional changes than E2 alone.

Conclusions

Patient-derived hEOs can be expanded long term while maintaining glandular epithelial characteristics, donor-specific transcriptional features, and hormone responsiveness. E2 and EPC induce distinct

transcriptional programs, with EPC producing a broader response that includes receptivity-associated molecular features. These findings support the use of hEOs as a physiologically relevant epithelial model for investigating hormonal regulation, endometrial receptivity, and reproductive disorders.

Introduction

The human endometrium is a highly dynamic and regenerative tissue that undergoes cyclic proliferation, differentiation, and secretion under the regulation of ovarian hormones, serving as a critical structure for the establishment and maintenance of pregnancy[1–3]. Dysfunction of endometrium associated with multiple disorders, including infertility, recurrent miscarriage, endometriosis, and endometrial atypical hyperplasia, which severely affect women's health[4]. Elucidating the biological characteristics and hormone-responsive of human endometrium is essential for exploring the endometrium-related diseases pathogenesis and drug development.

Traditional research of human endometrium mainly relies on in vivo animal models and monolayer single type cell culture systems. Increasing articles indicated that animal models restricted by species differences, cannot fully recapitulate the unique structural characteristics, hormone response patterns, and secretory functions [5]. And traditional monolayer or early co-culture systems fail to mimic the complex architectural features of the endometrium in vitro[6]. Up to now, no single model can simultaneously cover human-specific tissue architecture, hormonal cycles, immune and vascular components, and embryo-uterine interactions[7]. These inherent drawbacks have greatly restricted the in-depth investigation of endometrium dynamic physiological processes, limited the disease pathogenesis, and drug screening. It is urgent to construct a more ideal in vitro research model of human endometrium.

The development of endometrial organoids and related 3D systems stems precisely from their superior recapitulation of the structure and function of native endometrium. Human endometrial organoids can be expanded long-term and recapitulate the molecular, histological, and hormone-responsive characteristics of the original tissue[8]. Upon hormonal treatment, these models can also simulate menstrual cycle-related changes, including morphological and molecular characteristics of the secretory phase, and endometrial glandular organoids are capable of recapitulating glandular architecture and ultrastructure[9]. Organoids have become a core technical tool for tissue development research, disease mechanism analysis, and preclinical drug evaluation. Human endometrial organoids derived from clinical primary endometrial tissues possess the potential to retain the biological characteristics of native endometrium.

Most existing studies have only focused on the morphological construction of endometrial organoids, without systematic verification of their epithelial polarity, secretory functions, and hormone-induced cyclic differentiation characteristics, which greatly limits the application of endometrial organoids in the simulation of endometrial receptivity and the study of related pathological mechanisms[10, 11]. This study systematically established large-scale patient derived endometrial organoids, innovatively

integrates and solve widely concerned issues in 3D cultivation: the efficiency of organoid derivation, whether organoid epithelia exhibit proper polarity, whether secretions exist within the luminal space of organoids, whether human endometrial organoids are responsive to exogenous hormonal stimulation, what phenotypic responses are triggered by hormones, which genes and proteins undergo altered expression, and whether such hormonal induction can drive organoids toward a receptive-like endometrial state. Compared with traditional model research, our study recapitulates the morphological characteristics, biochemical functions, and hormone-dependent cyclic dynamic changes of human endometrium more detailed. The stable and verifiable hEOs model constructed in this work provides a reliable in vitro research platform for exploring the regulatory mechanism of endometrial receptivity, studying the pathogenesis of endometrial reproductive disorders, and screening potential therapeutic drugs, laying a solid foundation for subsequent basic research and clinical translational application of human endometrium-related diseases.

Methods

Human Endometrial Organoid Culture

Human endometrial tissues were collected using endometrial suction curettes. All patients provided written informed consent. This study was approved by the Institutional Ethics Committee (approval No. 2024-KY-1821-002). Endometrial organoids were established from freshly isolated endometrial tissues and cultured in a three-dimensional Matrigel matrix (Corning, USA). Organoids were maintained in endometrial organoid expansion medium (ExM) consisting of Advanced DMEM/F12 (Gibco) supplemented with B27 minus vitamin A (Gibco), N2 supplement (Gibco), GlutaMAX (Gibco), nicotinamide (Sigma-Aldrich), N-acetyl-L-cysteine (Sigma-Aldrich), A83-01 (Sigma-Aldrich), recombinant human EGF (Proteintech), Noggin (MedChemExpress), FGF10 (Proteintech), R-spondin-1 (Proteintech), HGF (Proteintech), and Primocin (InvivoGen). Medium was replaced every 2–3 days. Detailed protocols for organoid establishment, passaging, cryopreservation and recovery are provided in the Supplementary Materials and Methods.

Hormone Treatment

To evaluate hormone responsiveness and induce endometrial differentiation, organoids were treated with β -estradiol (E2), progesterone (P4) and cyclic adenosine monophosphate (cAMP). Following passaging, organoids were initially cultured in standard expansion medium (ExM) for 3 days. On day 3 (designated as experimental day 0, D0), wells were randomly assigned to one of three groups for a uniform 6-day evaluation window (9 days of culture in total after passage): untreated controls (NC), E2-treated organoids (E2), or E2/P4/cAMP treated organoids (EPC). Working concentrations were maintained at 10nM for E2, 1 μ M for P4, and 10 μ M for cAMP. The NC group was maintained in ExM without hormonal supplementation. To model the proliferative phase, the E2 group received ExM supplemented with 10nM E2 from D0 to D6 (6 consecutive days). To induce secretory differentiation,

organoids were primed with E2 alone for 2 days (D0–D2), followed by combined treatment with E2, P4 and cAMP for an additional 4 days (D2–D6). All organoids were harvested immediately on D6 for downstream functional and molecular analyses (Fig. 3A).

Histological Characterizations and Immunofluorescence

Regarding histological analysis, organoids and corresponding endometrial tissues were fixed in 4% paraformaldehyde, paraffin-embedded and sectioned. Haematoxylin and eosin (H&E) staining and periodic acid–Schiff (PAS) staining was performed using standard procedures.

For immunofluorescence analysis, paraffin sections underwent antigen retrieval, blocking and incubation with primary antibodies overnight at 4°C, followed by fluorescent secondary antibodies and DAPI nuclear counterstaining. The following primary antibodies were used for organoid characterisation and hormone-response analyses: EpCAM (ab282457, Abcam), MUC1 (ab28081, Abcam), wide-spectrum cytokeratin (ab9377, Abcam), E-cadherin (20874-1-AP, Proteintech), Laminin β 1 (23498-1-AP, Proteintech), oestrogen receptor α (ab108398, Abcam), progesterone receptor (ab101688, Abcam and 85112-4-RR, Proteintech), Ki67 (Abcam), PAEP (18187-1-AP, Proteintech), HSD β 2 (10978-1-AP, Proteintech), OPN (83341-1-RR, Proteintech). Images were acquired using a fluorescence microscope.

Morphological Analysis of Organoids

Organoid morphology was examined using GelNest Organoid Software (Nest Life Technology, China). Bright-field images were analyzed to determine organoid number, area, perimeter and circularity. Image scale calibration was performed using ImageJ software prior to analysis. Objects not corresponding to intact organoids were excluded before statistical evaluation. One-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test was used for statistical comparison.

Quantitative Real-Time PCR

Total RNA was extracted from organoids using TRIzol reagent (Invitrogen) and purified using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using PrimeScript RT Master Mix (Takara) according to the manufacturer's protocol. Quantitative real-time PCR was performed to assess the expression of endometrial receptivity-associated genes, including *PAEP*, *SPP1* and *HSD17B2*, using *GAPDH* as the internal reference gene. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences are provided in supplementary materials and methods.

RNA Extraction, Library Preparation, and Bulk RNA-Sequencing

Total RNA was extracted from organoids using TRIzol reagent and purified with the RNeasy Mini Kit (Qiagen). RNA integrity was assessed ($RIN \geq 8.0$) prior to library construction. Library preparation was performed using the Optimal Dual-mode mRNA Library Prep Kit (BGI-Shenzhen). Bulk RNA-seq library preparation and sequencing are provided in supplementary materials and methods. RNA-seq data have been deposited in GSA-Human (<http://ngdc.cncb.ac.cn/gsa-human>) under the accession number HRA020031[12, 13].

RNA-seq data analysis for different passage

Gene-level read counts from 18 organoid samples (6 groups $\times$ 3 replicates: Patient 1 P3/P4, Patient 2 P3/P5, Patient 11 P1/Patient 3 P2) were filtered to remove lowly expressed genes (total reads < 10), retaining 16,809 genes. Differential expression analysis was performed using DESeq2 (pydeseq2 v0.5.4) with three contrasts: Patient 1 P4 vs P3, Patient 2 P5 vs P3, and Patient 11 P1 vs Patient 3 P2. DEGs were defined as $|\log_2FC| > 1$ and adjusted $P < 0.05$ (Benjamini–Hochberg). Variance-stabilizing transformation (VST) was applied for PCA, Pearson correlation, and heatmap visualization. The top 10 DEGs (ranked by maximum $|z\text{-score}|$) were displayed in a clustered heatmap. Gene sets were classified as stable (non-DEG in all contrasts), common DEG (significant in ≥ 2 contrasts), or core identity (top-quartile expression, bottom-quartile CV). GO and KEGG enrichment was performed via Enrichr (gseapy) with adjusted $P < 0.05$. Figures were prepared to publication standards.

RNA-seq data analysis for hormone treatment

For the estrogen treatment experiment, uterine organoids from three patients (FL, GK, HLL) were treated with vehicle control (NC), 17β -estradiol (E2), or E2-P4-cAMP (EPC) for 6 days ($n = 3$ per group, 9 samples total). Gene-level read counts were filtered (total reads < 10), retaining 16,270 genes. Differential expression analysis was performed using DESeq2 (pydeseq2 v0.5.4) with two contrasts: NC vs E2 and NC vs EPC. The E2 vs EPC comparison yielded zero DEGs and was excluded from downstream analysis. Common DEGs were defined as the intersection of NC-vs-E2 and NC-vs-EPC DEGs with concordant directionality. VST normalization was applied for PCA and heatmap visualization. GO and KEGG enrichment was performed via Enrichr (gseapy) with adjusted $P < 0.05$. Heatmaps displayed the top DEGs ranked by $|\log_2FC|$, with samples ordered by treatment group (NC right, treatment left).

Ethics approval and consent to participate

Endometrial biopsy specimens used in this study were obtained under the approved ethics project entitled *“Investigating the role of the IL-6/JAK/STAT3 pathway in the pathogenesis of adenomyosis using organoid technology”*. Sample collection was approved by the Ethics Review Committee of the First Affiliated Hospital of Zhengzhou University on December 10, 2024 (approval No. 2024-KY-1821-002). All participating patients provided written informed consent for tissue collection and subsequent in-vitro experimental analyses. The present work focuses on establishing and characterizing patient-derived

human endometrial organoids for endometrial receptivity research using biospecimens collected within this approved project. All procedures were performed in accordance with institutional guidelines and local regulations.

Statistical Analysis

Data are presented as mean $\pm$ SEM. Statistical analyses were performed using GraphPad Prism 8. Comparisons between groups were performed using t-test or one-way analysis of variance (ANOVA). P values < 0.05 were considered statistically significant.

Results

Isolation and Primary Culture of hEOs

In this study, a total of 19 human endometrial samples were obtained via endometrial biopsy, and long-term and stable endometrial glandular organoids were successfully established from 18 samples, with a derivation efficiency of 94.7% (Supplementary Figure 1). After digestion and impurity removal, endometrial tissues were embedded in Matrigel to form microdrops and cultured in 24-well plates (Figure 1A). Elongated free endometrial glands were observed after tissue digestion, and small spherical three-dimensional structures were microscopically visible during continuous culture. These spherical structures gradually grew and expanded with prolonged culture time (Figure 1B). The human endometrial organoids (hEOs) maintained stable cell viability after continuous passaging, which could be passaged for more than 10 generations and cultured in vitro for over four months (Figure 1C) (Supplementary Figure 2). Mature hEOs cultured in vitro exhibited clear boundaries and typical translucent glandular luminal structures.

hEOs formed gland-like luminal structures and expressed glandular epithelial markers.

Morphological observation of hEOs was performed via hematoxylin and eosin (HE) staining. HE staining results revealed that hEOs presented typical glandular luminal structures lined with monolayer or pseudostratified epithelial cells. These cells were neatly arranged with oval nuclei located at the basal region, which was highly consistent with the glandular characteristics of native endometrial tissues (Figure 1D). The hEOs exhibited stable morphological features, with most organoids displaying vacuolar structures and a small number showing a compact state. Structurally, hEOs were composed of tightly arranged tall or short columnar or cuboidal epithelial cells. Periodic acid–Schiff (PAS) staining demonstrated prominent pink positive signals within the glandular lumens and the apical cytoplasm of partial epithelial cells, indicating the synthesis and secretion of glycogen or neutral mucopolysaccharides (Figure 1E). Collectively, the glandular luminal architecture verified by HE staining and the intraluminal glycogen accumulation detected by PAS staining confirm that the established hEOs not only recapitulate the anatomical structure of human endometrium but also possess the key biochemical functions of secretory-phase endometrium, including glycogen synthesis and storage. Immunofluorescence staining revealed robust expression of glandular epithelial markers including E-

cadherin, EpCAM, broad-spectrum cytokeratin (CK-5), MUC1 and Laminin β 1 in the cytoplasm and cell membrane of hEO (Figure 1F). Consistent with native endometrium, the basement membrane component Laminin β 1 was specifically localized at the basolateral side of epithelial cells in hEO, whereas MUC1 was restricted to the luminal surface. These spatial distribution patterns indicate the establishment of fundamental epithelial polarity in hEO (Figure 1G).

Inter-generational and inter-individual variabilities of hEOs

All 18 samples showed high transcriptomic concordance (mean Pearson $r = 0.970$; biological triplicates $r > 0.98$) (Figure 2A), confirming culture reproducibility. PCA separated samples primarily by patient identity (PC1, 36.5%) and secondarily by passage (PC2, 21.3%) (Figure 2B).

DESeq2 analysis identified 4,134 unique DEGs across three comparisons: Patient 1 P3→P4 (1,489 DEGs, 9.4%), Patient 2 P3→P5 (2,615 DEGs, 16.2%), and Patient 11 P1 vs Patient 3 P2 (1,765 DEGs, 10.9%). The longer passage interval (P3→P5) yielded nearly twice as many DEGs as the shorter interval (P3→P4), with a disproportionate increase in downregulated genes (1,659 vs 729), indicating progressive transcriptional silencing with extended culture (Figure 2C-H). Differentially expressed genes identified within the same patient are frequently associated with inflammation (*IL1B*), cell adhesion (*CEACAM*), and cytoskeleton-related pathways (*TUBA1A*), whereas the differentially expressed gene *HLA-DQA1* indicates that organoids exhibit patient-specific unique characteristics. (Figure 2F-H).

Despite these changes, 75.4% of genes (12,675) remained stable across all comparisons, forming a robust transcriptional core. Only 288 common DEGs were recurrently altered in ≥ 2 comparisons. We next characterized the shared differentially expressed genes across serial-passaging comparisons of patient-derived endometrial organoids. KEGG pathway enrichment analysis revealed prominent enrichment of immune- and inflammation-related pathways, including the IL-17 signaling pathway, NOD-like receptor signaling pathway, TNF signaling pathway, and cytokine-cytokine receptor interaction (Figure 2I). Consistent with KEGG results, GO-biological process enrichment further highlighted antiviral immune defense responses, alongside extracellular matrix organization (Figure 2J).

In summary, organoids bring patient-specific characteristics, maintaining a highly stable transcriptional core across passages with passage-dependent changes primarily affecting immune and inflammatory programs.

Hormonal stimulation increased the number and size of hEOs

To construct an in vitro endometrial receptive model, hormonal stimulation was applied to mimic the cyclic changes of the endometrium and observe the dynamic alterations of hEOs. hEOs derived from two patients were treated with estradiol (E2), progesterone (P4), and cyclic adenosine monophosphate (cAMP). E2 supplementation was initiated on day 0 (D0) in both the E2 and EPC groups, whereas P4 and cAMP were added to the EPC group from day 2 (D2). The negative control (NC) group received no hormone treatment (Figure 3A). The number and cross-sectional area of organoids were demarcated

and quantified on day 6 (D6) with Organoid Software (Figure 3B-C). The number of Ki-67-positive cells in hormone-treated hEOs increased without statistical significance (Supplementary Figure 3A).

For patient 1, significant differences in organoid number were detected among the three groups ($F(2,12) = 9.737$, $p < 0.01$). Compared with the NC group, both the E2 group ($p = 0.0043$) and EPC group ($p = 0.0101$) exhibited markedly increased organoid counts (Figure 3C c-1). Significant differences in average organoid area were also observed among groups ($F(2,12) = 33.32$, $p < 0.0001$), with a remarkable increase in the E2 group ($p < 0.0001$) and EPC group ($p = 0.0001$) (Figure 3C c-2). For patient 2, no significant difference in organoid number was found across the three groups ($F(2,6) = 2.288$, $p = 0.1826$) (Figure 3C c-3). However, the average organoid area differed significantly among groups ($F(2,6) = 30.35$, $p = 0.0007$), and the average area was significantly larger in the E2 group ($p = 0.0008$) and EPC group ($p = 0.0028$) (Figure 3C c-4). Collectively, these results demonstrate that treatment with estrogen and progesterone effectively promoted the proliferation and enlargement of hEOs.

Hormone-treated hEOs expressed receptive markers

The expression profiles of endometrial receptive markers were further examined in hormone-treated hEOs derived from two patients. Progestagen-associated endometrial protein (PAEP) is a glandular epithelial-specific protein directly regulated by progesterone, with significantly higher levels in the mid-to-late secretory phase than in the late proliferative phase[14]. Secreted phosphoprotein 1 (SPP1) is a key epithelial differentiation marker and a component of glandular secretions closely associated with endometrial epithelial secretory activity and is upregulated during the late secretory and decidualization phases[15]. 17-beta-hydroxysteroid dehydrogenase type 2 (HSD17B2) is a progesterone-regulated enzyme that catalyzes the conversion of estradiol to inactive estrone in human endometrial epithelial cells, and its expression is elevated in the late secretory and decidualized endometrium[15]. Estrogen acts through estrogen receptor α (ER α /ESR1) to target estrogen-response elements on the *PGR* gene promoter and upregulates endometrial PR expression[16]. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to detect the expression of these receptive markers following hormonal stimulation. The results showed that *PAEP* was constitutively expressed in all groups. Compared with the NC group, the expression levels of *PAEP*, *SPP1*, and *HSD17B2* were markedly increased in both the E2 and EPC groups (Figure 3D). Immunofluorescence confirmed the expression of SPP1, HSD17B2 and PAEP expressed in endometrial organoids, and progesterone receptor (PR) in endometrial organoids following E2 and EPC treatment (Figure 3E-F and Supplementary Figure 3B-C).

Hormone-treated hEOs exhibit a secretory-phase transcriptional profile.

We further performed bulk RNA-seq on NC, E2 and EPC organoids from 3 patients to explore whether they can recapitulate endometrial receptivity in vitro. PCA of VST-normalized counts separated samples primarily by treatment (PC1, 58.3%), with NC samples clustering distinctly from E2 and EPC samples. PC2 (17.7%) reflected inter-patient variability. E2 and EPC samples overlapped almost completely in PCA space, indicating highly similar transcriptomic responses. (Figure 4A). All samples showed high pairwise correlation (Pearson $r = 0.93$ -1.00) (Figure 4B). E2 and EPC stimulation induced partially overlapping yet

distinct transcriptional reprogramming patterns in hEOs. DESeq2 identified 1341 DEGs in NC vs E2 (919 up, 421 down) and 1517 DEGs in NC vs EPC (1013 up, 504 down) (Figure 4C-D). Volcano plots showed 421 downregulated and 919 upregulated DEGs in the E2 group relative to NC, while 504 downregulated and 1013 upregulated DEGs were found in the EPC group versus NC. *SERPINA3*, *S100P*, *TFPI2* and *KCNG1* were significantly upregulated in both E2 and EPC groups. These genes regulate stromal protease activity and modulate endometrial stromal degradation as well as epithelial integrity. Genes specifically upregulated in the EPC group included *ARL4D*, *ENPP3* and *COL1A2*, while *AOC1* represented a prominent downregulated gene shared between the two treatment groups. The specific upregulation of *COL1A2* upon combined hormone treatment suggests that progesterone participates in the regulation of extracellular matrix remodelling. Collectively, EPC treatment induced more DEGs than E2 treatment alone, demonstrating that combined estrogen and progesterone leads to more profound transcriptomic reprogramming in endometrial organoids (Figure 4E-F). To explore the biological functions of common DEGs upon EPC stimulation in human endometrial organoids, GO-BP and KEGG enrichment analyses were performed. GO biological process enrichment revealed that these shared DEGs were predominantly enriched in cilia-related biological processes, including *cilium movement* (n=36), *cilium assembly* (n=50), *axoneme assembly* (n=24), and *axonemal dynein complex assembly* (n=22). Other enriched terms involved microtubule bundle formation and determination of left-right symmetry. KEGG pathway analysis indicated that common DEGs were mainly enriched in pathways including amoebiasis (n=19), protein digestion and absorption (n=18), AGE-RAGE signaling pathway in diabetic complications (n=16), ECM-receptor interaction (n=15), as well as multiple amino-acid metabolic pathways such as arginine and proline metabolism. Collectively, these findings suggested that hormone stimulation of endometrial organoids robustly modulates cilia-associated biological processes together with extracellular matrix interaction and amino-acid metabolic signaling (Figure 4G-H).

Discussion

In this study, we successfully constructed a stable in vitro culture system of human endometrial glandular organoids (hEOs) with a derivation efficiency of 94.7%. The constructed hEOs can be stably passaged for more than 10 generations and cultured in vitro for over four months as previously reported[8,17]. Morphological and histological identification showed that hEOs exhibited typical glandular luminal structures, with neatly arranged monolayer or pseudostratified epithelial cells and basal-localized oval nuclei consistent with human endometrial tissues. PAS staining and immunofluorescence secretion and glandular epithelial markers expression and polarity. Transcriptomic confirmed the stability of the hEO model. Limited passage-dependent transcriptional variations were mainly enriched in immune and inflammatory pathways, without altering the core endometrial epithelial identity, while retained patient-specific molecular characteristics. EPC treatment significantly upregulated the expression of endometrial receptivity marker genes *PAEP*, *SPP1* and *HSD17B2* in hEOs, and PR was induced by E2 treatment. Transcriptomic analysis identified differentially expressed genes following E2 and EPC treatment. *SERPINA3*, *S100P*, *TFPI2* and *KCNG1* were uniformly elevated under both interventions, while E2 treatment specifically upregulated *CXCL1* and *ALPP*, EPC treatment

specifically upregulated *ARL4D*, *ENPP3* and *COL1A2*. Collectively, these data reveal distinct hormone-driven transcriptional signatures governing proliferative and receptive states of hEOs, laying a foundation for further exploration of endometrial regulatory networks and receptivity biomarkers.

Estrogen and progesterone are the core regulatory hormones of endometrial cyclic changes, which jointly mediate endometrial proliferation, secretory differentiation and receptivity acquisition[18,19]. Hormone responses of patients' endometrium progressed slowly due to limitations of repeated biopsy. In-vitro-cultured hEOs largely overcame this limitation. Statistical results showed that hormone treatment significantly increased the number and cross-sectional area of hEOs, which is consistent with the in vivo physiological rule that estrogen dominates endometrial proliferation and progesterone promotes endometrial maturation[8]. Stably established organoid lines also ensure high experimental reproducibility. In the present study, two E2- and EPC-induced genes (*SERPINA3* and *S100P*) identified via bulk RNA-seq have been reported in previous endometrial research. But most studies focused on tumor cells. The positive expression rate of *SERPINA3* was 71.25% in endometrial carcinoma tissues, markedly higher than the 31.25% observed in normal endometrial tissues[20]. Mechanistically, *SERPINA3* facilitates proliferation and suppresses apoptosis of endometrial carcinoma cells via regulating the G2/M cell cycle checkpoint, and its pro-proliferative effect may stem from activation of the MAPK/ERK1/2 and PI3K/AKT signaling pathways[21]. As an acute-phase inflammatory protein, *SERPINA3* expression is regulated by IL-1 and IL-6 cytokines via the STAT3 pathway[22]. *S100P* is also markedly overexpressed in endometrial carcinoma tissues and cooperates with multiple S100 family members including *S100A2*, *S100A7* and *S100A9* to drive endometrial carcinogenesis. As intracellular Ca²⁺-binding sensors and extracellular mediators, S100 protein family modulates cellular behaviors, and their dysregulated expression correlates with proliferation, invasion, migration and drug resistance of gynecological malignancies[23]. *S100A4* is estrogen-regulated and serves as a downstream target of estrogen-related receptor γ (ERR γ); upregulated ERR γ positively promotes *S100A4* transcription[23]. However, direct evidence supporting hormonal regulation of *S100P* remains lacking. *TFPI2* and *KCNG1* identified in this study may represent novel stably expressed estrogen-stimulated genes in endometrium, which requires further investigation. This study first demonstrated that these genes can be induced upon estrogen-progesterone stimulation in human-derived endometrial organoids, providing evidence for its subsequent investigation as an estrogen and progesterone responsive gene and for studies on endometrial receptivity.

The transformation of the endometrium from the proliferative to the secretory phase remains poorly investigated due to difficulties in clinical sampling. Accurate identification of the endometrial receptive window and targeted diagnosis of recurrent implantation failure are critical for embryo transplantation in assisted reproductive technology, yet specific biomarkers for endometrial receptivity are still under continuous exploration. In this study, three significantly upregulated genes (*ARL4D*, *ENPP3*, and *COL1A2*)

were identified in endometrial organoids following in vitro EPC treatment. Among them, ENPP3 acts as a strictly progesterone-dependent biomarker for endometrial receptivity, which is highly expressed exclusively in glandular epithelium during the implantation window and secreted into uterine fluid in glycosylated form. In vitro functional assays have directly established a causal link between ENPP3 and human blastocyst attachment[24]. This study firstly verified that *ENPP3*, a progesterone-specific receptive biomarker of human endometrium, could be normally induced upon EPC treatment in hEOs. *COL1A2* acts as a canonical extracellular matrix gene whose exclusive induction confirms progesterone-mediated ECM reconstruction[25], while the functional roles of *ARL4D* and *COL1A2* in endometrial receptivity remain unelucidated. This could be the focus of our future research.

The endometrium is a highly dynamic mucosal tissue lining the uterine cavity, which undergoes periodic proliferation, differentiation and shedding driven by ovarian steroid hormones, and is the core tissue supporting embryo implantation and early pregnancy maintenance in humans[26]. Endometrial glands, as key functional components of the endometrium, mediate embryonic nutrient supply, immune regulation and endometrial decidualization, and are indispensable for successful pregnancy establishment[27–29]. Abnormal endometrial cyclic remodeling and glandular dysfunction are closely associated with infertility, recurrent implantation failure, endometrial hyperplasia and endometrial cancer, severely affecting women's reproductive health. However, the in-depth mechanistic study of human endometrial physiology and pathological changes has long been restricted by the lack of reliable in vitro models. Traditional research models have prominent limitations. Immortalized or carcinoma-derived endometrial cell lines possess transformed phenotypes and cannot faithfully recapitulate the physiological structure and hormone responsiveness of normal endometrial tissues[30]. Primary endometrial cells are difficult to sustain long-term in vitro culture, rapidly lose original phenotypes and hormone sensitivity after passage, and the clinical endometrial biopsy samples are limited in quantity and non-expandable[31]. Murine models cannot perfectly mimic human endometrial cyclic changes due to the species difference that mice have an estrous cycle rather than a menstrual cycle[32]. Compared with conventional two-dimensional cell culture systems and animal models, three-dimensional endometrial organoid can better simulate the in vivo histological structure, cellular polarity and functional characteristics of the endometrium, which gradually became an ideal tool for endometrial research[8]. The organoids directly isolated from endometrial tissues in early studies lacked long-term expandability and complete phenotypic identification, and could not be used for in-depth functional research[33,34]. Compared with previously reported hEOs, this study has obvious advantages in stability and simulation authenticity. Not only fully validates that hEOs generated by this culture method recapitulate endometrial morphology, cellular secretion and epithelial polarity in vitro with consistency, but also alongside stability during long-term passaging. We uncover the potential of hEOs as models for endometrial receptivity and pathological conditions and make sequencing datasets publicly available as a reference for subsequent hormone-related endometrial research. Nevertheless, despite improved structural and hormonal responsiveness, current 3D endometrial organoid systems cannot fully recapitulate critical features such as immunity, vascularization, and luminal polarity[35].

Nevertheless, this study still has certain limitations. First, the current hEOs model only contains endometrial glandular epithelial components, lacking stromal cells, immune cells and vascular components in native endometrial tissues. Although the single epithelial organoid can simulate the hormone response and secretory function of endometrial epithelium, it cannot fully reproduce the complex cell-cell interaction in the in vivo endometrial microenvironment, resulting in a slightly weaker hormone response effect than in vivo tissues[36]. Second, this study only verified the morphological and molecular changes of hEOs in simulated menstrual cycle stages, and the specific molecular mechanisms of hormone regulation of organoid proliferation and differentiation still need to be further explored.

In conclusion, this study successfully constructed a patient derived stable, long-term expandable human endometrial glandular organoid model, which can faithfully simulate the morphological structure, biochemical function and hormone response characteristics of native endometrium. The hormone-treated hEOs can effectively simulate the formation of endometrial receptive state and stably express key receptive marker genes. This high-fidelity in vitro model makes up for the defects of traditional research models, provides a reliable technical platform for exploring the molecular mechanism of human endometrial cyclic remodeling and receptivity regulation, and lays a foundation for the research and development of targeted drugs for infertility and endometrial diseases, as well as the clinical application of endometrial regenerative repair.

Declarations

Ethics statement

This study was conducted as part of the approved project entitled “Investigating the role of the IL-6/JAK/STAT3 pathway in the pathogenesis of adenomyosis using organoid technology.” The project was approved by the Ethics Review Committee of the First Affiliated Hospital of Zhengzhou University on December 10, 2024 (No. 2024-KY-1821-002). Written informed consent was obtained from all participants. The study was conducted in accordance with local legislation and institutional requirements.

Data Availability Statement

RNA-seq raw data have been deposited in the GSA-Human database under accession number HRA020031. The minimal datasets supporting the conclusions of this study are available from the corresponding author upon reasonable request.

Author Contributions Statement

M.Z. conceived and designed the study, drafted the main manuscript text, and acquired the principal funding. M.T. performed the organoid culture and molecular biology experiments. Y.X. assisted with the

organoid culture and molecular biology experiments. W.S. and H.Y. contributed to the experimental design and performed immunofluorescence staining. H.Z. performed RNA sequencing and bioinformatics analyses. H.W. and J.W. collected the clinical samples. L.H. contributed to the experimental design and acquired additional funding. All authors reviewed and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing financial interests

Declarations

The authors declare that they have not used AI-generated work in this manuscript.

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Figures

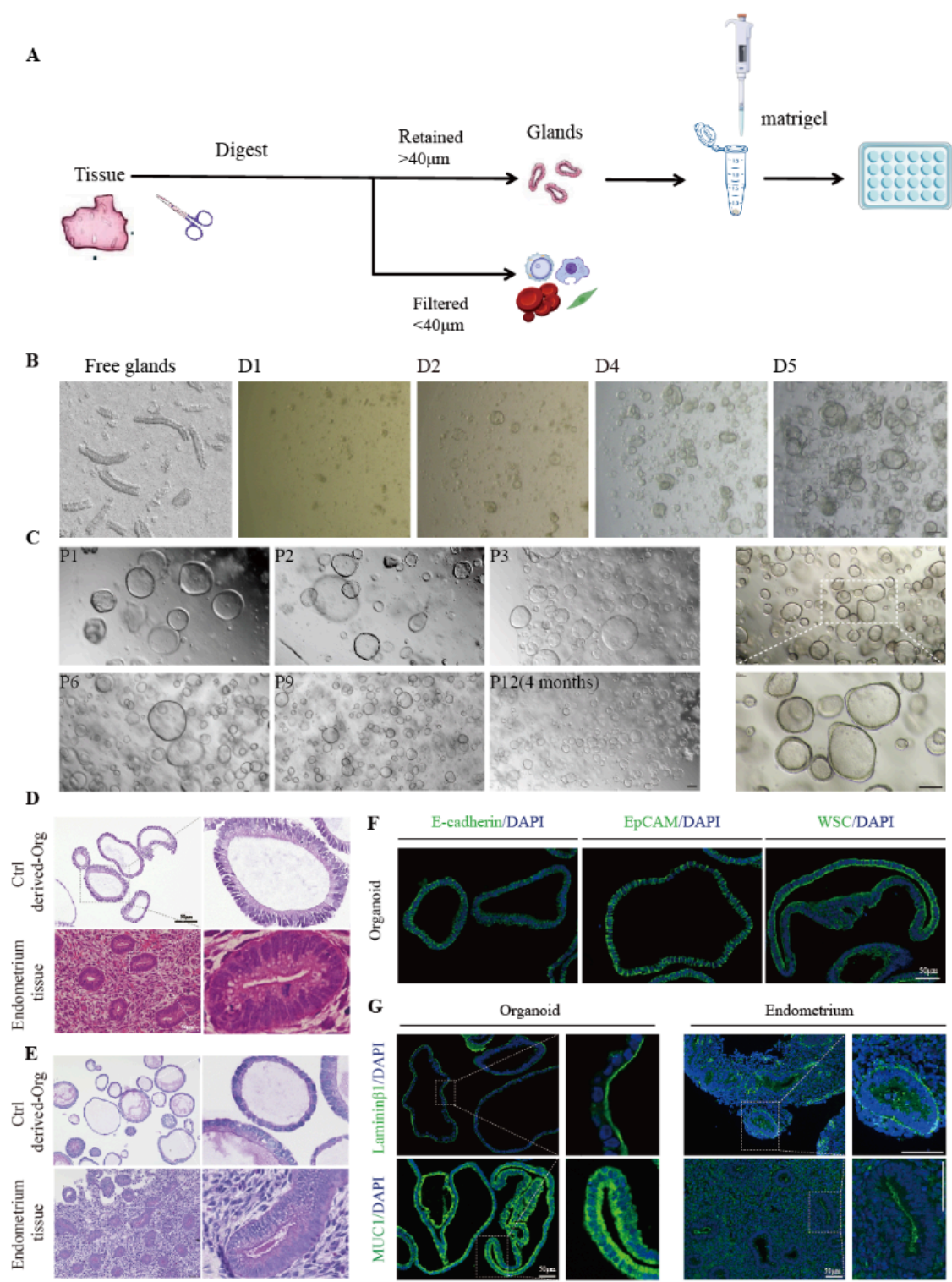


Figure 1

Isolation, establishment and culture of endometrial organoids

(A) Schematic diagram illustrating the establishment of tissue-derived hEOs.

(B) Morphology of hEOs. Gland-like structures with clear boundaries and obvious translucency can be observed after 4–5 days of culture.

(C) Morphology of normal hEOs at passages 1 to 12 (P1–P12) during subculture. hEOs maintained stable and consistent morphological features with spherical glandular architecture throughout long-term culture. And light microscopic images of endometrial organoids at magnifications of 40× and 100×. Scale bar = 100 µm.

(D) HE staining of hEOs and endometrial tissues. The organoids exhibited glandular luminal structures lined mainly by monolayer or pseudostratified epithelia with regularly arranged cells. Nuclei were located at the basal side of the cells, consistent with the glandular architecture of native endometrial tissues.

(E) PAS staining of normal hEOs and endometrial tissues. Pink positive granular signals were detected inside the organoid lumens and in the apical cytoplasm of partial epithelial cells, indicating glycogen synthesis and secretion. Scale bar = 50 µm.

(F) Staining of glandular epithelial markers in normal hEOs. E-cadherin, EpCAM and wide-spectrum cytokeratin (CK-5) were strongly expressed.

(G) Immunofluorescence staining of Laminin β 1 and MUC1 in hEOs and endometrial tissues. The basement membrane component Laminin β 1 was distributed at the basolateral side, while MUC1 localized to the luminal side. Similar to native endometrial tissues, the organoids established fundamental epithelial polarity. Scale bar = 50 µm.

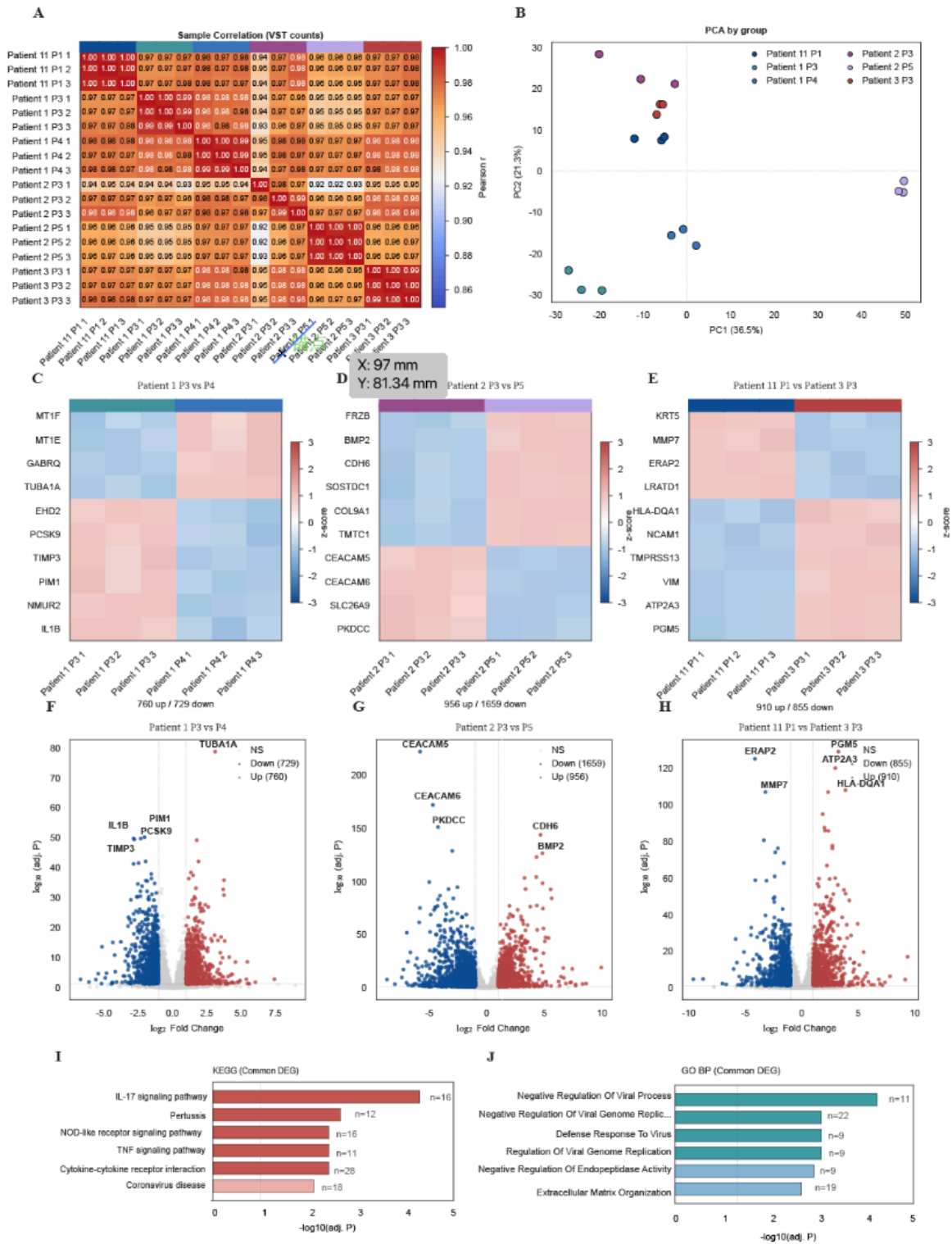


Figure 2

Transcriptomic analysis of hEOs.

(A) Pearson correlation heatmap. (B) PCA of 18 samples (top 2,000 variable genes, VST). (C) DEG heatmap (top 30 genes, z-scored VST). (D,E) GO BP and KEGG enrichment of 288 common DEGs (top 8 terms). (F) Volcano plots for three comparisons (red: up, blue: down, grey: NS; top 7 DEGs labeled).

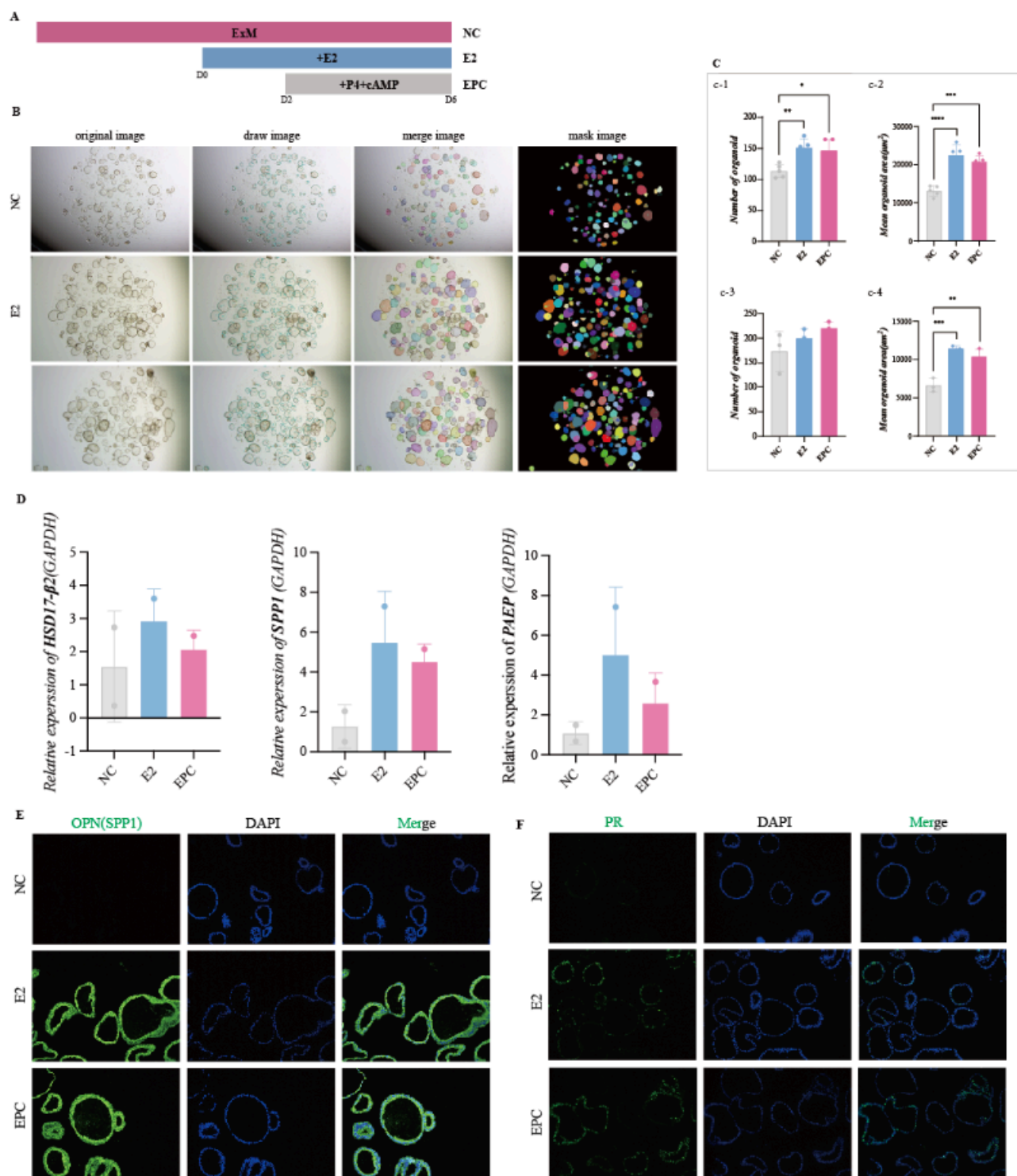


Figure 3

Alterations of hEOs following hormone treatment

(A) Schematic diagram of hormone treatment protocol for organoids. After routine passaging, hEOs were cultured in organoid expansion medium (ExM) for 3–4 days prior to hormone administration.

(B) Representative images of endometrial organoids analyzed by GelNest Organoid Software after hormone intervention.

(C) Bar graphs showing organoid count and average area (total area/count) in each treatment group.

(D) Relative expression levels of receptivity-related genes (*PAEP*, *SPP1*, and *HSD17B2*) detected by real-time PCR after hormone treatment (n = 3).

(E) Representative immunofluorescence images for OPN(*SPP1*) and PR in NC/E2/EPC groups.

Data are presented as mean $\pm$ SEM. Each plot corresponds to organoids derived from an individual patient donor (Donor 1: c-1, c-3; Donor 2: c-2, c-4), and each dot denotes measurements obtained from a single well. *p < 0.05, **p < 0.01, ***p < 0.001 versus NC group (Tukey's HSD test).

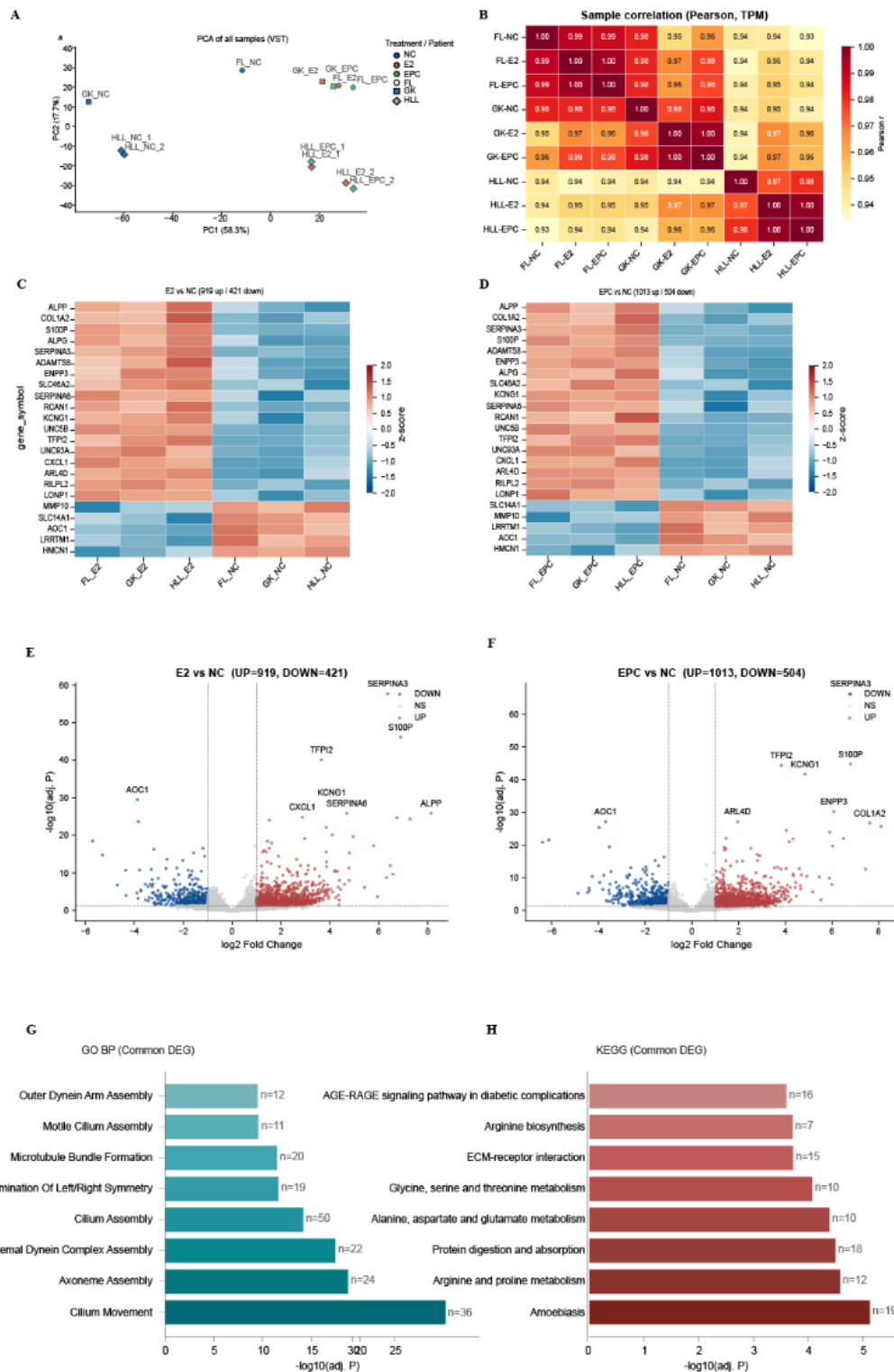


Figure 4

Transcriptomic profiling of endometrial organoids under E2 and EPC treatment.

(A) PCA (VST): PC1=58.3%, PC2=17.7%; colors=treatment (NC/E2/EPC), shapes=patient (FL/GK/HLL). (B) Sample correlation (Pearson, TPM; $r=0.93-1.00$, $n=9$). (C) DEG heatmap E2 vs NC (919 up/421 down; z-score). (D) DEG heatmap EPC vs NC (1,013 up/504 down). (E) Volcano E2 vs NC (red=up, blue=down,

gray=NS; labeled *SERPINA3/S100P/AOC1/TFPI2/KCNG1/CXCL1/SERPINA6/ALPP*). (F) Volcano EPC vs NC (labeled *SERPINA3/S100P/TFPI2/KCNG1/ENPP3/AOC1/ARL4D/COL1A2*). (G) GO BP enrichment (common DEGs n=1,303; cilium movement/axoneme assembly top). (H) KEGG enrichment (amoebiasis/arginine metabolism/AGE-RAGE top). Statistics: DESeq2 (Wald, BH); hypergeometric enrichment (BH); Pearson on TPM.

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