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Supplementary Information for

**Capture and metabolomic analysis of the human endometrial epithelial organoid secretome**

Constantine A. Simintiras, Pramod Dhakal, Chaman Ranjit, Harriet C. Fitzgerald, Ahmed Z. Balboula, and Thomas E. Spencer

Corresponding author: Thomas E. Spencer
Correspondence**:** spencerte@missouri.edu

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**Other supplementary materials for this manuscript include the following:**

Movie S1
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## Supporting Materials and Methods

### EEO Culture.

EEO were prepared similarly to Fitzgerald *et al*. (1). In brief, upon collection, endometrial biopsies were placed in DMEM/F12 medium (Gibco 11320-033) supplemented with 10% charcoal-stripped Fetal Bovine Serum and 1% antibiotic-antimycotic (ABAM; Gibco 15240-062) and transported to the laboratory within 1 h of surgery. Endometrial biopsies were then washed in DMEM/F12 supplemented with 1% ABAM, finely minced manually, and subsequently enzymatically digested by incubation in DMEM/F12 supplemented with 1% ABAM, 0.4 mg/ml Collagenase V (Sigma C-9263), and 1.25 U/ml Dispase II (Sigma D4693), at 37 °C with orbital agitation for ~ 50 m. Neutralizing medium – consisting of DMEM/F12 with 1% ABAM and 10% Fetal Bovine Serum (FBS; ThermoFisher, 16000036) – was then added before passing the digestion through 100-µm cell strainers. The 100-µm cell strainers were inverted, and glandular fragments/epithelial cells were forcefully backwashed and pelleted by centrifugation at 270 x *g* for 10 m prior to pellet resuspension in 1 ml Advanced DMEM/F12 (Gibco, 12634010). Cells were then re-centrifuged and incubated on ice for 2 min prior to resuspension in Matrigel (Corning 536231) at a dilution of 500,000 viable cells per ml. Matrigel-suspended endometrial epithelia were seeded to 48-well plates via the addition of one 20 µl Matrigel dome (*i.e.*, 10,000 cells) per well. These plated cells were incubated at 37 °C for 15 m before being overlaid with 250 µl pre-equilibrated organoid expansion culture medium (OEM) per well, identically to Fitzgerald *et al*. (1). Organoid formation ensued by their maintenance at 37 °C with 5% $CO_2$ in air. Cultures were expanded by passaging prior to their cryopreservation, involving the manual transfer of confluent Matrigel domes, using a 1 ml wide-boar micropipette, to a 15 ml tube for centrifugation at 270 x *g* for 10 m at 4 °C. The pellet was resuspended in 5 ml chilled Advanced DMEM/F12 + 1% ABAM, dissociated by pipetting up and down and re-centrifuged prior to resuspension in 10% DMSO in FBS at a volume equivalent to 30,000 cells per ml. EEO were transferred to cryovials at 1 ml per vial and stored at -80°C for 24-48 h before transfer to $N_2(l)$ for long-term storage.

For experiments, EEO were thawed by serial resuspension with pre-equilibrated OEM. This mixture was then centrifuged at 270 x *g* for 10 m and the pellet resuspended in sufficient Matrigel to achieve 500,000 viable cells per ml. Matrigel-suspended endometrial epithelia were seeded to 48-well plates via the addition of one 20 µl Matrigel domes (*i.e.*, 10,000 cells) per well. Cells were incubated at 37 °C for 15 m before the addition of 750 µl pre-equilibrated OEM supplemented with 10 µM Y27632 (Peprotech, 1293823) per well for the first three media changes. Y27632 was excluded from subsequent media changes. Spontaneous organoid formation occurred following 3-4 d of culture at 37°C under 5% $CO_2$ in air.

### Time-lapse Imaging of EEO Formation.

EEO fragments from donors 1, 2, and 3 – at their respective $13^{th}$, $10^{th}$, and $8^{th}$ passage – were transferred and plated to a single 35 mm glass-bottomed petri-

dish (MatTek, P35G1.0-14C). Immediately after, the dish was stabilized within a microenvironmental chamber maintaining an atmosphere of 5% $CO_2$ and temperature of 37°C in humidified air. Images of select cell fragments were taken using a 40x air objective on a Leica DMI8 microscope at 30 m intervals for 60 h with 5 to 10 µm z-intervals under minimal light exposure and intensity. A representative movie is provided as supplementary material (Mov. S1) with representative images in Fig. 1B.

**EEO Metabolomics – MMN vs. HTC.**

Untargeted metabolomic analysis of IOF derived by MMN *vs*. HTC in addition to CM (*i.e.,* Fig. 2) was performed the University of Missouri Metabolomics Core, as described by Mao *et al*. (2). In brief, 1 ml 100% methanol was added to every 50 µl of sample prior to vigorous vortex and sonication for 20 s each. Samples were then incubated at -20 °C for 1 h before the addition of 20 µl 1 mg/mL aqueous ribitol to each sample. Samples were vortexed for 5 s before centrifugation at 13,000 x *g* for 15 mins. One (1) ml of supernatant was transferred to a labelled autosampler vial and dried under a gaseous nitrogen stream. Dried sample extracts were then methoximated by the addition of 30 µl pyridine supplemented with 15 mg/ml methoxyamine hydrochloride and incubation for 1 h at 50 °C. Samples were then trimethylsilylated by the addition of 30 µl N-methyl-N-(trimethyl-silyl)-trifluoroacetamide (MSTFA) + 1% chlorotrimethylsilane (TMCS) reagent, and incubated for a further 1h at 50 °C. These derivatized extracts were transferred to 150 µl inserts within autosampler vials and run on an Agilent 6890 GC coupled to a 5973N MSD MS with a scan range of 50-650 m/z (Agilent Technologies, Santa Clara, CA, USA). Chromatography was achieved by injecting and passing 1 µl sample through a 60 m, 0.25 mm ID, 0.25 µm film thickness DB-5MS GC column (J&W Scientific, Folsom, CA, USA) at a constant flow rate of 1.0 ml/min with helium gas at a split ratio of 1:1. Peak separation was achieved using the following temperature gradient: 80 °C for 2 m ramping to 315 °C for 12 m at a rate of 5 °C per m. For quality control and retention index calculations, a standard alkane mix was used in addition to ribitol as an internal standard in each sample, to which relative concentrations were normalized. Chromatographic data were deconvoluted using AMDIS and annotated by mass spectral and retention index matching to an in-house library. Unidentified spectra were matched to the commercial NIST17 library. Final processing was performed using custom MET-IDEA software.

**EEO Metabolomics – IOF vs. EOF.**

Ultrahigh performance liquid chromatography tandem mass spectroscopy (UPLC-MS/MS) of HTC-derived IOF, EOF, CM, and MCM (*i.e.*, Figs. 3 and 4) was performed by Metabolon Inc. (Durham, NC, USA) as previously described in Simintiras *et al*. (3–6). Briefly, any protein was precipitated and extracted using the automated MicroLab STAR system (Hamilton Company) with methanol under vigorous centrifugation at 680 *x g* for 2 min (Geno/Grinder 2000, Glen Mills) prior to methanol removal using a TurboVap (Zymark) and overnight incubation in nitrogen. Each sample was subsequently divided into 4 fractions – two for analysis by reverse phase (RP) UPLC-MS/MS with positive ion mode electrospray ionization (ESI), one for analysis by RP UPLC-MS/MS with negative

ion mode electrospray ionization (ESI), and one for analysis by hydrophilic interaction liquid chromatography (HILIC) UPLC-MS/MS with negative ion mode ESI. Sample extracts were then dried and reconstituted in solvents compatible to each UPLC-MS/MS procedure. Specifically, the first fraction analyzed under positive ionization was subject to gradient elution (Waters UPLC BEH 1.7 µm C18 column 2.1 x 100 mm) in water and methanol with 0.05% perfluoropentanoic acid and 0.1% formic acid. The second run under positive ESI was identically eluted, using the same column, but with the elution buffer additionally comprising acetonitrile. The third fraction, analyzed under negative ionization, was similarly eluted using a gradient buffer comprising methanol, water, and 6.5 mM ammonium bicarbonate (pH 10.8), and the fourth ran under negative ESI and was eluted using a HILIC (Waters UPLC BEH Amide 1.7 µm column 2.1 x 150 mm) with a water plus acetonitrile plus 10 mM ammonium formate (pH 10.8) gradient. Samples were subsequently analyzed using a Waters Acquity UPLC coupled to a Thermo Scientific Q-Exactive high resolution MS interfaced with heated electrospray ionization (HES-II) source and Orbitrap mass analyzer operating at 35,000 mass resolution and with a scan range between 70-1000 *m*/*z*. Biochemicals were quantified against known internal and recovery standards, run in parallel at random intervals. Metabolite identification was based on retention time and a *m*/*z* within ± 10 ppm. The technical (instrument) median relative standard deviation was 5% with a total process variability of 10%.

**EEO Metabolomic Data Normalization and Presentation.**
Data normalizations for MMN-IOF *vs*. HTC-IOF and HTC-IOF *vs*. EOF Are provided in Datasets S1 and S2, respectively. For semi-quantitative HTC-derived IOF and EOF metabolomic analyses, data values were imputed where readings were partially incomplete (Dataset S2). Unless otherwise stated, metabolomic data were compared by two-way ANOVA with a $P \leq 0.05$ denoting significance and $0.05 < P < 0.10$ denoting a trend. Biochemical networks (Fig. 4H) were visualized using MetaboLync™ pathway analysis software. Principal components analyses were conducted using the open-access Past4 software (7). Additional statistics and heat-mapping – details of which are provided within corresponding figure legends – were generated using Prism 8 (GraphPad, San Diego, CA, USA).

**EEO Population and Size Analyses.**
Brightfield EEO images were taken on days 0 and 6 of culture (Fig. 1F-G) using an upright Evos XL Core microscope (Thermofisher, Waltham, MA, USA) at 40X combined magnification. Images were taken at random locations from 4 wells per donor. EEO numbers (count) and size (area) were manually respectively counted and measured using NIH ImageJ (v. 1.51). Units were converted to $\mu m^2$ before mean (n=12 per day) and standard error contrasting by one-way ANOVA and a Tukey's multiple comparison *post hoc* using Prism 8 (GraphPad, San Diego, CA, USA)

**EEO RNA Isolation and Sequencing.**
Following IOF extraction by HTC, residual endometrial epithelial cell pellets were suspended in 1 ml Trizol reagent (Invitrogen, 15596026) and stored at -80 °C

until further processing. Thereafter, tubes were thawed on ice and vigorously vortexed for 2 min for homogenization, and 300 µl chloroform per 1 ml Trizol was added to each tube and mixed well. This mixture was then passed through 5PRIME heavy-gel phase-lock tubes (Andwin Scientific, 10847802) by centrifugation at 14,000 x *g* for 7 min at 4 °C and the supernatant was processed using the Direct-zol RNA extraction protocol (Zymo Research, R2072) according to manufacturer instructions. Purified RNA was quantified by Qubit (Invitrogen) and quality was assessed using an Agilent 2100 Bioanalyzer. All RNA samples had an RNA integrity number (RIN) value ≥ 9.0. Bulk RNA sequencing was performed similarly to Fitzgerald *et al*. (1). RNA library preparation and sequencing were conducted by Novogene (Sacramento, CA, USA). Raw sequences (FASTQ) were subjected to quality check by *FastQC* and the *fqtrim* program was used to remove adapters. Quality trimming was performed using a *phred* threshold of >30, a sliding 6 nucleotide window scan, and select read length of ≥30 nucleotides or longer post-trimming. Reads were mapped to the human reference genome (GRCh38) using the Hisat2 aligner. Relative expression was calculated using *featureCounts* and converted to fragments per kilobase of transcript per million mapped reads (FPKM) values in *edgeR*. Transcriptomic raw data is available in the Gene Expression Omnibus (GSE166289). Transcriptomic profiles were visualized and compared by one-way analysis of variance (ANOVA) coupled to the Geisser-Greenhouse correction and the Tukey's multiple comparisons *post hoc* using Prism 8 (GraphPad, San Diego, CA, USA). *P*-value summaries are provided in corresponding figures and figure legends. Gene ontology enrichment analysis (Dataset S3) was performed using the open-access gene ontology PANTHER overrepresentation test with an FDR $P<0.05$ cut-off as determined by a Fisher's exact significance test (8, 9). Metabolic gene transcript FPKM extrapolation to metabolic pathway flux (Fig. 5) was performed using the open-access Pathview rendering platform (10, 11) coupled to the KEGG database (12).

**Dataset S1 (separate file)**. *Micromanipulation vs. High-throughput centrifugation intra-organoid fluid raw and processed metabolomic data*. Metabolomic profiles of organoid expansion culture medium (CM) and intra-organoid fluid (IOF) obtained by high-throughput centrifugation (HTC) *vs*. micromanipulation (MMN) from each donor (D). Data were normalized three times, firstly accounting for GC/MS sample loading (technical normalization), secondly accounting for biological parameters, as described in the manuscript proper (biological normalization) and thirdly, logarithmically transformed to correct for skewness (statistical normalization).

**Dataset S2 (separate file)**. *Quantitative and Qualitative intra-organoid (IOF) vs. extra-organoid fluid (EOF) raw and processed data.* Metabolomic profiles of IOF and EOF obtained from each donor (D). Under the *raw* columns, metabolite presence is indicated by green cells, whereas their absence is grey. Under the *ANOVA* columns, cell colors reflect metabolite fold changes, where red denotes a significant ($P \leq 0.05$) increase, pink depicts a trend ($0.05 < P \leq 0.10$) towards an increase, dark green depicts a decrease ($P \leq 0.05$), and light green represents a trend towards a decrease. Additional statistical parameters are also provided. Asterisks denote predicted metabolites.

**Dataset S3 (separate file)**. *Gene ontology representation test.* Testing was conducted using all transcripts from all donors. The 20 most positively over-represented, arranged by false discovery rate (FDR), are highlighted.

**Supplementary Movie 1 (separate file)**. *EEO formation.* Representative time-lapse imaging of an endometrial epithelial organoid from donor 1, at a static z-stack, forming after being passaged. Images were taken at 30 min intervals for 60 h.

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