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An improved method for isolation of epithelial and stromal cells from the human endometrium

Ayako MASUDA[1, 2], Noriko KATOH[1, 2], Kazuhiko NAKABAYASHI[1)], Kiyoko KATO[3)], Kenzo SONODA[3)], Mari KITADE[2)], Satoru TAKEDA[2)], Kenichiro HATA[1)] and Junko TOMIKAWA[1)]

[1)]*Department of Maternal-Fetal Biology, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan*
[2)]*Department of Obstetrics and Gynecology, Faculty of Medicine, Juntendo University, Tokyo 113-8431, Japan*
[3)]*Department of Obstetrics and Gynecology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-0054, Japan*

Abstract. We aimed to improve the efficiency of isolating endometrial epithelial and stromal cells (EMECs and EMSCs) from the human endometrium. We revealed by immunohistochemical staining that the large tissue fragments remaining after collagenase treatment, which are usually discarded after the first filtration in the conventional protocol, consisted of glandular epithelial and stromal cells. Therefore, we established protease treatment and cell suspension conditions to dissociate single cells from the tissue fragments and isolated epithelial (EPCAM-positive) and stromal (CD13-positive) cells by fluorescence-activated cell sorting. Four independent experiments showed that, on average, 1.2×10^6 of EMECs and 2.8×10^6 EMSCs were isolated from one hysterectomy specimen. We confirmed that the isolated cells presented transcriptomic features highly similar to those of epithelial and stromal cells obtained by the conventional method. Our improved protocol facilitates future studies to better understand the molecular mechanisms underlying the dynamic changes of the endometrium during the menstrual cycle.

Key words: Cell separation, Endometrium, Endometrial epithelial cells (EMECs), Endometrial stromal cells (EMSCs), Gene expression array

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The endometrium, the inner layer of the uterus, is essential for successful reproduction. It becomes receptive to implanting blastocysts during the implantation window and forms the maternal-fetal interface with the placenta during pregnancy. Dysfunction of the endometrium is involved in pathologies responsible for female infertility, such as implantation failure and recurrent pregnancy loss. The endometrium undergoes a cycle of regeneration, proliferation, differentiation and desquamation several hundred times during the reproductive period from menarche to menopause under the control of the ovarian steroidal hormones, estrogen and progesterone. Although gene expression patterns associated with these hormone-dependent morphological and functional changes of the endometrium during the menstrual cycle are considered to be at least partially epigenetically regulated, information on epigenetic regulation in the endometrium has so far been limited [1, 2]. The remarkable regenerative capacity of the endometrium is understood to involve the presence of endometrial stem/progenitor cells. Therefore, the endometrium is regarded as an excellent source of mesenchymal stem cells for regenerative medicine [3, 4].

The endometrium is mainly composed of fibroblastic stromal and glandular epithelial cells. Decidualization of endometrial stromal cells and various factors secreted by the glandular epithelium are crucial for embryo implantation and development and maintenance of pregnancy. Protocols for the isolation of endometrial stromal and epithelial cells (EMSCs and EMECs) have been well established [4–6]. However, although it is easy to obtain a large number of EMSCs by cell culture due to their high proliferative potential, the number of EMECs obtainable by current protocols is relatively small. Consequently, it has been difficult to subject EMECs to the types of analyses that require a large number of cells. Here, we report a modified method by which we were able to isolate more than 1×10^6 of both EMECs and EMSCs with over 95% purity reproducibly from a single total hysterectomy case without a long-term cell culture.

We initially isolated EMECs and EMSCs from the endometrium using the conventional protocols [5–7] with slight modifications (Fig. 1A). Microphotographs of EMEC and EMSC fractions seeded on tissue culture dishes (at 0 h and 36 h) are shown in Fig. 1B. The cell clumps after the second filtration mainly contained tubular-shaped EMEC clusters (upper left in Fig. 1B, 0 h). The EC clusters gradually adhered to the bottom surface of the dish and formed flat and extended cell colonies (lower left in Fig. 1B, 36 h). The filtrate fraction after the second filtration presumably contained suspended single cells of EMSCs, EMECs and blood cells. Due to different proliferative potentials depending on cell types, EMSCs were selectively proliferated through a long-term culture in a medium containing 10% FBS.

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Correspondence: K Hata (e-mail: hata-k@ncchd.go.jp) or J Tomikawa (e-mail: tomikawa-j@ncchd.go.jp)
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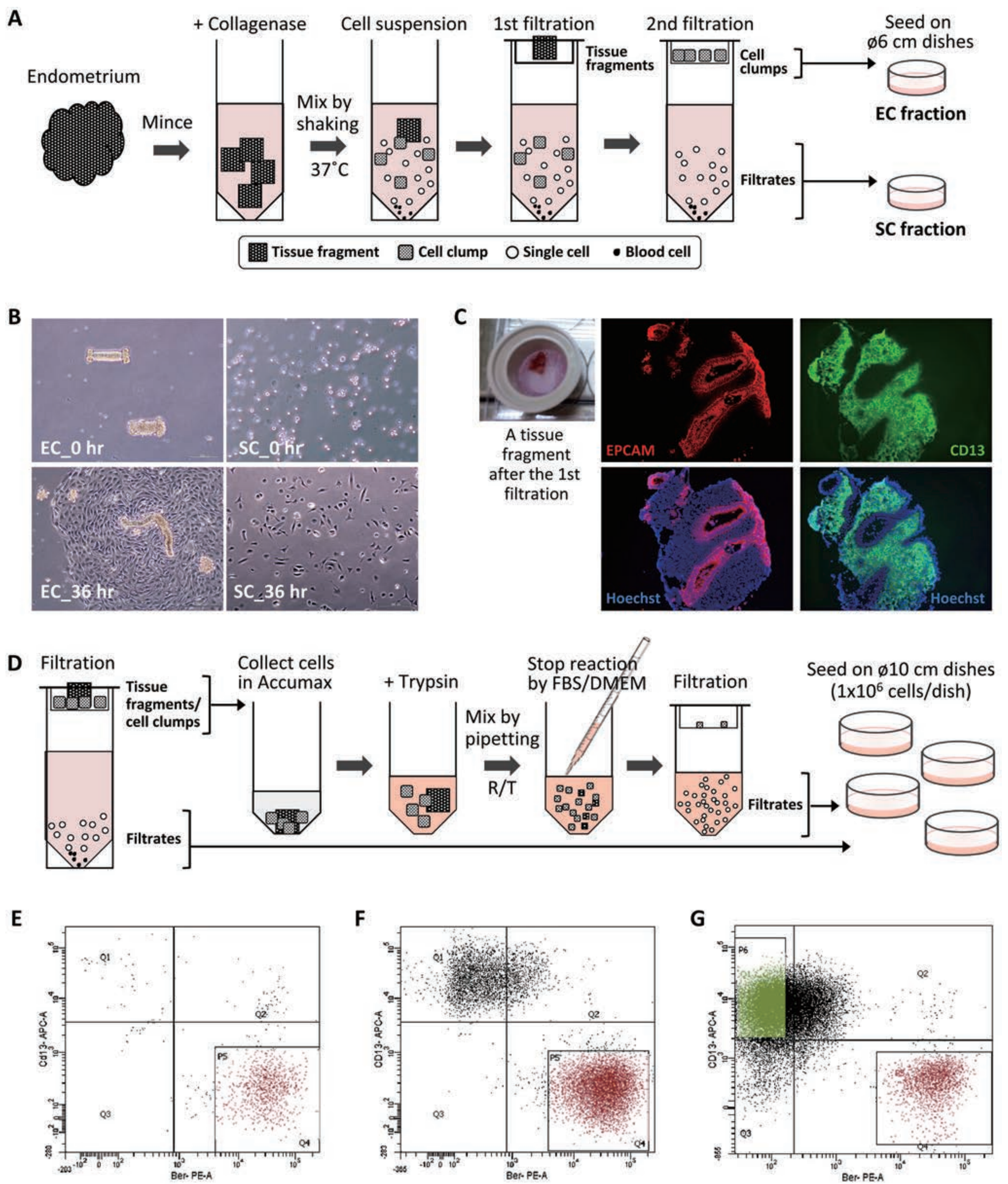

Fig. 1.

Table 1. Clinical information of donor patients, cell isolation protocol, and numbers of epithelial and stromal cells (ECs and SCs) isolated from the endometrium (EM)

Case ID [a]	Age (years)	Diagnosis	Operation [b]	Thickness of EM (mm)	Menstrual phase [c]	# of ECs obtained [d]		# of SCs obtained [e]		RNA sample ID in Fig. 2
						Conventional protocol	Modified protocol	Conventional protocol	Modified protocol	
EM1008	28	Dermoid cyst	LC	13.5	P	-	-	2.10×10^7 (4 passages)	-	1008_P_SC_conv
EM0206	40	Myoma	ATH	-	P	-	-	6.27×10^7 (4 passages)	-	0206_P_SC_conv
EM0625	29	Dermoid cyst	LC	4.8	S	-	-	2.40×10^7 (4 passages)	-	0625_S_SC_conv
EM0128	31	Dermoid cyst	LC	9.0	P	-	-	-	-	
EM0808a	23	Mucinous	LC	4.0	P	1.68×10^3	2.50×10^4	-	-	0808a_P_EC_conv 0808a_P_EC_modified
EM0808b	30	Mucinous	LC	4.6	P	2.03×10^3	2.90×10^4	-	-	0808b_P_EC_conv 0808b_P_EC_modified
EM0723	23	Dermoid cyst	LC	8.8	S	-	7.40×10^4	-	-	
EM0827	23	Dermoid cyst	LC	7.0	S	-	4.20×10^4	-	-	
EM1018	47	Myoma	ATH	-	P	-	1.55×10^6	-	4.00×10^6	1018_P_SC_modified 1018_P_EC_modified
EM1206	46	Myoma, dermoid cyst, EM polyp	ATH	-	P	-	1.50×10^6	-	2.00×10^6	
EM1016	49	Myoma	ATH	-	S	-	1.20×10^6	-	2.70×10^6	1016_S_SC_modified 1016_S_EC_modified
EM0416	50	Myoma	ATH	-	S	-	0.65×10^6	-	2.50×10^6	

[a] No donor patients used exogenous hormone therapy before surgery. [b] ATH, abdominal total hysterectomy; LC, laparoscopic cystectomy. [c] P, proliferative; S, secretory. [d] The ECs were isolated by FACS at 48–96 h after seeding on a 6-cm dish without passage. [e] The SCs isolated by the conventional protocol were expanded through four serial passages during cell culture and counted.

Although over 1.0×10^7 EMSCs were obtained from a single donor, after four passages with > 90% purity consistently, the numbers of EMECs isolated tended to be much lower, typically under 1.0×10^4, as were the cases for EM0808a and 0808b (Table 1).

We revealed by immunohistochemistry that the residual tissue fragments after the first filtration (Fig. 1A), which are usually discarded in the conventional protocol, consisted of glandular epithelial and stromal cells: the antibody against an epithelial cell marker, epithelial cell adhesion molecule (EPCAM) [6], stained glandular epithelial structures, whereas the antibody against a stromal cell marker, CD13 [7], stained the surrounding regions of the glandular epithelia (Fig. 1C). Therefore, we established protease treatment and cell suspension conditions (as described in the Methods section), which enabled us to dissociate single cells from the residual endometrial tissue fragments. The ratios of the live cells among the cells isolated by the established conditions ranged from 20% to 35%. By adopting these conditions, we developed a modified protocol to isolate single cells from endometrial tissues (Fig. 1D). In this protocol, the isolated single cells were seeded on collagen I-coated dishes and cultured for a few days. Subsequently, the cells were subjected to immunostaining using anti-EPCAM and anti-CD13 antibodies conjugated with fluorescent proteins followed by fluorescence-activated cell sorting (FACS) to isolate epithelial (EPCAM-positive) and stromal (CD13-positive) cells. The flow cytometry dot plots represent the increase in the number of EMECs (EPCAM-positive cells) isolated by the modified protocol (Fig. 1F and G) compared with the number of EMECs isolated by the conventional protocol (Fig. 1E), and they show the presence of two major clusters (EPCAM-positive cells and CD13-positive cells) in the single cells isolated by the modified protocol (Fig. 1F and G).

In two specimens (EM0808a and EM0808b), we compared the

Fig. 1. Isolation of EMECs and EMSCs by conventional and modified protocols. A) A schematic outline of the conventional protocol. Detailed procedures are described in the Methods. B) Phase-contrast microscopy images of the isolated endometrial cells obtained by the conventional protocol: EMEC and EMSC fractions at 0 h and 36 h. C) Immunohistochemical detection of EMECs (EPCAM-positive cells) and EMSCs (CD13-positive cells) in a residual tissue fragment after the first filtration (Case ID: EM0128). EPCAM and CD13 staining only (upper panels) and merged with nuclear staining with Hoechst 33342 (lower panels). D) A schematic outline of the modified endometrial cell preparation protocol. Detailed procedures are described in the Methods. E) Flow cytometry dot plots for FACS-sorted EMECs (EPCAM-positive cells) isolated by the conventional protocol (EM0808a) and F) those isolated by the modified protocol (EM0723). G) FACS-sorted EMECs and EMSCs (CD13-positive cells) isolated by the modified protocol from the endometrium of an ATH case (EM1018). Horizontal and vertical axes represent the florescent intensities of PE (conjugated with the anti-CD13 antibody) and APC (conjugated with the anti-EPCAM antibody), respectively. The rectangular areas P5 and P6 indicate the gates for sorting EMSCs (CD13-positive and EPCAM-negative cells) and EMECs (EPCAM-positive and CD13-negative cells), respectively.

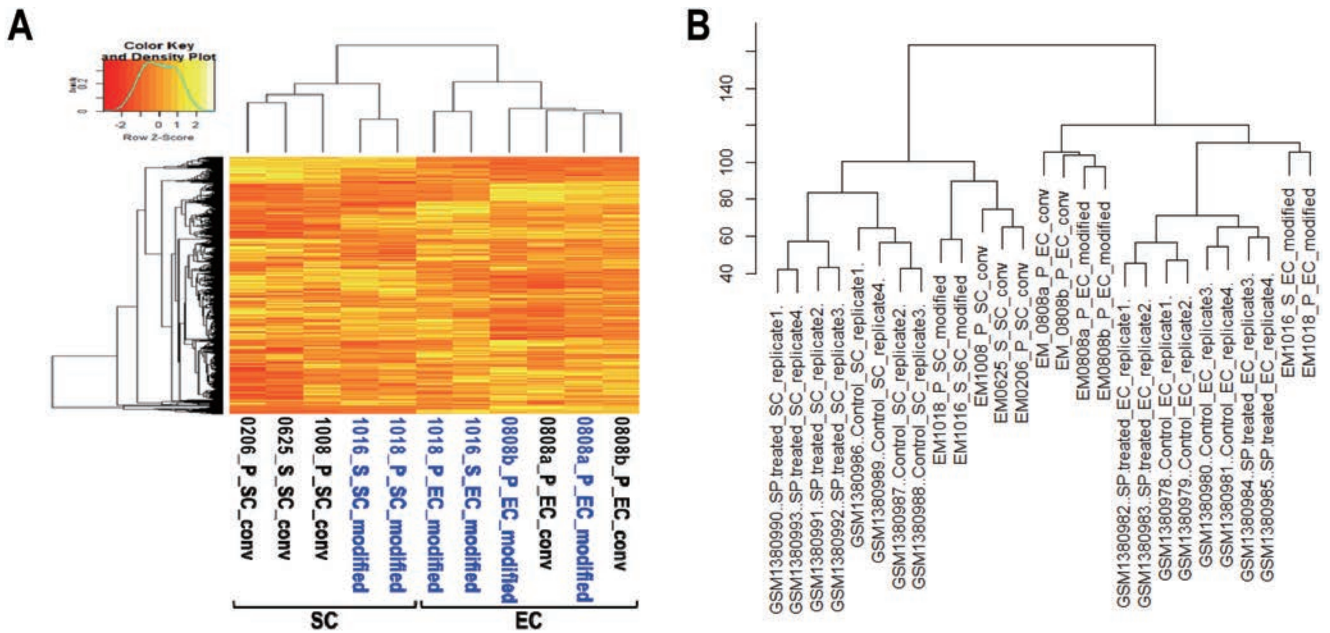

Fig. 2. Cluster analyses of microarray-based transcriptome data. A) Hierarchical clustering analysis of the expression profiles of EMSCs and EMECs isolated by the conventional and modified protocols in this study. The IDs underneath the heatmap contain information for donor ID, menstrual phase (proliferative (P) or secretory (S)), cell type (SC or EC) and the isolation protocol. B) Hierarchical clustering analysis of the expression profiles of EMSCs and EMECs prepared in this study and those (NCBI GEO accession number: GSE57356) prepared previously by a different group [8].

numbers of EMECs isolated by the conventional and modified protocols. These endometrial specimens were initially subjected to EMEC isolation using the conventional protocol. The tissue fragments and cell clumps after the first and second filtrations, which are usually discarded in the conventional protocol, were subjected to the modified protocol. In both cases, as shown in Table 1, the numbers of EMECs isolated by the modified protocol were approximately 14 times higher than those by the conventional protocol. Based on these results, which we reproduced in duplicate, we concluded that our modified protocol outperformed the conventional protocol in terms of the number of isolated cells. On average, four independent experiments showed that 1.2×10^6 of EMECs and 2.8×10^6 EMSCs were isolated from one hysterectomy specimen (Table 1, Case IDs EM1018, EM1206, EM1016 and EM0416).

We validated whether the cells isolated by our modified protocol possessed the characteristics of EMECs and EMSCs by conducting a microarray-based transcriptome analysis. Among the five EMSC samples and six EMEC samples tested, two and four samples were isolated by the modified protocol, respectively (Table 1). In a hierarchical clustering analysis of the 20,997 genes that were detected as expressed in the 11 samples, the phylogenic tree was branched into two large clusters of EMECs and EMSCs (Fig. 2A). Within the EMSC cluster, the cells isolated by the conventional protocol and those by the modified protocol were separated into different clusters. This is likely due to the difference in culture time (3–4 weeks versus a few days) and passage numbers (4 times *vs*. none) for EMSCs between the two protocols. In the EMEC cluster, the samples were not separated by the isolation protocols but rather were separated in a manner largely dependent on the donor individuals. These results demonstrate that the cells isolated by the modified protocol developed in this study maintain the transcriptomic properties of endometrial epithelial and stromal cells. We also compared our dataset with the transcriptome dataset GSE57356 (in the NCBI's Gene Expression Omnibus (GEO) database), which was obtained for EMECs and EMSCs by a different group [8]. In the hierarchical clustering analysis of the 18,676 genes with expression data in both datasets, the tree was branched into the EMEC and EMSC clusters (Fig. 2B). The branching pattern indicates that the transcriptomic properties of EMECs and EMSCs obtained in our study are generally similar to those of cells in the GSE57356 study.

In this study, we developed a protocol to isolate at least 10 times more EMECs than the conventional protocol. Our modified protocol also enabled us to obtain a large number of EMSCs without long-term cell culture and passages. Although it is easy to expand the number of EMSCs through serial passages (e.g., $> 10^8$ cells), the cells may lose their original characteristics at least partially during *in vitro* cell culture. Because our modified protocol isolates EMSCs without cell passages, the isolated cells are expected to better maintain their *in vivo* characteristics in the endometrium.

While our modified protocol is expected not to solve the issue of EMSC contamination after long term culture of EMECs, it enabled us to isolate more than 10^6 EMECs with > 90% purity from the endometrium of a single total hysterectomy specimen. We plan to subject the isolated cells to chromatin immunoprecipitation and sequencing (ChIP-seq) analyses for six types of target histone modifications (H3K4me3, H3K27ac, H3K4me1, H3K36me3,

H3K9me3 and H3K27me3) designated by the International Human Epigenome Consortium (IHEC) to generate the first reference epigenome information for endometrial epithelial cells. In this consortium, use of highly purified cells as materials for epigenome analyses is encouraged [9]. Our improved protocol will help further expand knowledge and understanding about the molecular (and epigenetic) mechanisms underlying the functional dynamics of the endometrium during the menstrual cycle.

Methods

Endometrial tissues

Human endometrial tissues (n = 12) were collected from women (ranging from 23 to 50 years old) who underwent surgeryhad an operation (abdominal total hysterectomy (ATH, n = 5) or laparoscopic cystectomy (LC, n = 7)) due to myoma, dermoid cyst or mucinous tumor and did not use exogenous hormone therapy before surgery. The endometrial tissues were collected partially in LC cases (using an endometrial suction curette, Pipet Curet (MX140, CooperSurgical, Trumbull, CT, USA)) and entirely in ATH cases. Preoperative informed consent was obtained from each patient. This study was approved by the ethics committees of the National Research Institute for Child Health and Development, Juntendo University and Kyushu University. The phases of the menstrual cycle were categorized for each sample as the proliferative phase (n = 7) and the secretory phase (n = 5) based on the first day of the last menstrual period. The endometrium was scraped off and collected for examination, and the collected endometrial samples were kept in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) at 4˚C, and subjected to cell isolation procedures within 24 h.

Isolation and culture of human endometrial cells by the conventional protocol (Fig. 1A)

Endometrial tissues were minced into pieces of less than 1 mm in DMEM containing 50 U/ml penicillin, 50 mg/ml streptomycin and 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA). Minced tissues were incubated with 200 µg/ml collagenase NB4 (SERVA, Heidelberg, Germany) for 2 h at 37˚C with agitation. The dispersed endometrial tissue fragments/cells were separated into two fractions by filtration using a nylon mesh with 440-µm diameter pores (Costar, #3480, Corning Inc, Corning, NY, USA), the residual tissue fragments and the filtrate. This first filtrate fraction was further separated into two fractions by filtration using a nylon mesh with 40-µm diameter pores (#0556, BD Falcon, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), the residual cell clumps and the second filtrate. The residual cell clumps, which were considered to be pieces of glandular epithelia, were seeded onto a collagen I-coated 6-cm tissue culture dish (#345501, Corning) with MF-start medium (code:TMMFS-001, Toyobo, Osaka, Japan) as the EMEC fraction. The dispersed cells in the second filtrate were collected by centrifugation and seeded onto a collagen I-coated 6-cm tissue culture dish with MF-start medium as the EMSC fraction. Both groups of cells were maintained at 37°C in a humidified environment with 5% CO_2 in air. The EMEC outgrowths were trypsinized, collected, and counted for their cell numbers at 5 days after seeding. The EMSCs were expanded through four serial passages to $> 1.0 \times 10^7$ cells. Cells were collected by trypsinization and subjected to immunostaining followed by FACS.

Isolation of human endometrial cells by the modified protocol (Fig. 1D)

First, the mincing of endometrial tissues and collagenase treatment were conducted in the same way as in the conventional protocol (Fig. 1A). Then, the dispersed endometrial tissue fragments/cell clumps were separated into two fractions by filtration using a nylon mesh with 40-µm diameter pores: the residual tissue fragments/cell clumps and the filtrate corresponding to the SC fraction in the conventional protocol. The cells in the SC fraction were collected by centrifugation ($200 \times g$ for 5 min), resuspended in 1 to 2 ml of MF-start medium and kept at room temperature until seeding on tissue culture dishes. The residual tissue fragments and cell clumps were collected into a new 50 ml tube using 3 to 5 ml of Accumax (Innovative Cell Technologies, San Diego, CA, USA), which is a solution of proteolytic and collagenolytic enzymes, added to an equal volume of 0.25% Trypsin/EDTA (Gibco, catalog no. 25200-056, Thermo Fisher Scientific, Grand Island, NY, USA) and then incubated for 10 min at room temperature with continuous pipetting (or mild agitation). The resultant cell suspension was added with 30 ml of DMEM containing 10% FBS (SH30070, HyClone) to inhibit proteolytic and collagenolytic activity in the enzymes. After filtration using a nylon mesh with 40-µm diameter pores, the cells in the filtrate were collected by centrifugation ($200 \times g$ for 5 min), resuspended in MF-start medium and combined with the SC fraction cells. After the ratio of viable cells was determined by a trypan blue absorption assay using a portion of cells, the cells were seeded onto 10-cm collagen I-coated culture dishes (approximately 1×10^6 cells/dish), and maintained at 37°C in a humidified environment with 5% CO_2 in air. After 48–96 h, the adherent cells were dissociated by TrypLE Express Enzyme (Gibco, catalog no. 12604-013, Thermo Fisher Scientific), collected and subjected to immunostaining followed by FACS.

Immunohistochemistry of endometrial tissue fragments

Endometrial tissue fragments, the residues after the first filtration (Fig. 1A and C), were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan). Frozen sections (6 µm thick) of the embedded tissue were prepared using a cryostat, fixed with acetone at –20˚C for 10 min and rinsed twice with 1 × PBS for 5 min each. Frozen section slides were incubated with 3% BSA/1 × PBS for 1 h at room temperature (for blocking) in a humidified chamber followed by antibody reaction. To immunohistochemically detect the epithelial cell-specific marker, EPCAM, frozen section slides were incubated with a monoclonal antibody against EPCAM (product no. M080401, Dako, Carpinteria, CA, USA) (1:200 diluted) or with a non-immune mouse IgG (catalog no. 12-371, EMD Millipore, Billerica, MA, USA) as a negative control for 1h, and subsequently incubated with a secondary antibody, Alexa 647-conjugated anti-mouse IgG (product no. 4410, Cell Signaling Technology, Danvers, MA, USA) (1:1000 diluted), for 1 h. To detect the stromal cell-specific marker, CD13, frozen section slides were incubated with an anti-human CD13 antibody conjugated with allophycocyanin (APC) (1:60 diluted) (catalog no. 561698, BD Pharmingen, Becton, Dickinson and Company), or mouse IgG conjugated with APC (1:60 diluted) (catalog

no. 550854, BD Pharmingen, Becton, Dickinson and Company) as a negative control, for 1 h. Slides were subsequently washed with 1xPBS, counterstained with 20 µg/ml Hoechst 33342 dye (product no. B2261, Sigma-Aldrich, St. Louis, MO, USA) and mounted with glycerol. All immune reactions were conducted at room temperature. The fluorescent signals of the bound antibodies were examined using a fluorescence microscope system (Axio Imager 2, Carl Zeiss Microscopy, Jena, Germany) and ZEN (Carl Zeiss Microscopy) imaging software.

Isolation of human endometrial epithelial and stromal cells by immunostaining followed by FACS

Adherent cells were dissociated from the surface of the culture dishes using TryPLE Express (Gibco, catalog no. 12604-013, Thermo Fisher Scientific), collected, resuspended in 500 µl of 1 × PBS, passed through a 40-µm filter and divided into 450-µl and 50-µl cell suspensions. The cells in each of the suspensions were collected by centrifugation (200 × *g*, 5 min) and resuspended in 200 µl of 1 × PBS containing 2% BSA. Five microliters each of phycoerythrin (PE)-conjugated antibody against human EPCAM (LS-C112552, LifeSpan Biosciences, Seattle, WA, USA) and APC-conjugated anti-CD13 mouse monoclonal antibody (catalog no. 561698, BD Pharmingen, Becton, Dickinson and Company) were added to the former, and IgG controls (PE Mouse IgG1 Kappa Isotype Control (catalog no. 551436, BD Pharmingen, Becton, Dickinson and Company) and APC Mouse IgG1 Kappa Isotype Control (catalog no. 550854, BD Pharmingen, Becton, Dickinson and Company)) were added to the latter. The cell suspensions were incubated on ice for 30 min under light shielding. After washing with 1 × PBS twice, the cells were subjected to flow cytometry analysis and cell sorting using a FACS Aria II system (BD Biosciences). The EPCAM- or CD13-positive cells, corresponding to EMECs and EMSCs, respectively, were sorted based on the flow cytometry data.

Gene expression array analysis

Total RNA was extracted using an AllPrep Micro Kit (Qiagen, Hilden, Germany). Total RNA samples were subjected to gene expression microarray analysis using a SurePrint G3 Human GE Microarray 8x60K Kit (product no. G4851A, design ID 28004, Agilent Technologies, Santa Clara, CA, USA) by following the manufacturer's instructions. This array contains a total of 50,599 probes covering 27,958 RefSeq genes and 7,419 lincRNAs. Total RNAs (5 ng each) were amplified using Ovation Pico WTA System V2 (NuGen Technologies, San Carlos, CA, USA) and labelled with Cy3 using a Low Input Quick Amp Labeling Kit (Agilent). The resultant cRNAs were fragmented at 60°C for 30 min in the dark, and 600 ng of them were hybridized to the microarray at 65°C for 17 h. After washing, slides were scanned with an Agilent G2565CA Microarray Scanner. Feature Extraction Software (version 10.7.3.1) was used to calculate signal intensities from the scanned images. Raw intensity data were imported into the Subio Platform software (https://www.subio.jp/) and normalized (75th percentile global normalization). The hierarchical clustering analyses were performed using heatmap.2 in the R package gplots (http://cran.r-project.org/web/packages/gplots/gplots.pdf), and hclust (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/hclust.html), with complete linkage with Euclidean distance as parameters. The expression array dataset has been deposited in the NCBI GEO and is accessible through GEO Series accession number GSE73090.

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