

Isolation and Culture of Human Endometrial Epithelial Cells and Stromal Fibroblasts

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[Abstract] Purification and culture of endometrial epithelial cells (eEC) and stromal fibroblasts (eSF) from endometrial biopsies allows for downstream cell-specific *in vitro* studies. The utility of this protocol is the ease with which cells are purified without contamination from unwanted cell types, and the ability to use patient-paired eEC and eSF in experiments. These methods have been previously published, but here the protocol has been updated for maximum efficiency.

Materials and Reagents

1. Falcon™ 15 ml Conical Centrifuge Tubes (Thermo Fisher Scientific, catalog number: 14-959-49D)
2. Cell Culture/Petri Dishes (100 x 20 mm) (Thermo Fisher Scientific, Nunc™, catalog number: 172958)
3. Falcon™ 50 ml Conical Centrifuge Tubes (Thermo Fisher Scientific, catalog number: 14-432-22)
4. Costar® 24 Well Clear TC-Treated Multiple Well Plates, Bulk Pack, Sterile (Corning, catalog number: 3527)
5. Sterile Filtration Units (0.1 µm pore) (Merck Millipore Corporation, catalog number: SCVPU02RE)
6. Procurement of endometrial biopsies
 - a. Endometrial biopsies from reproductive age women (ages 28–53) were obtained as previously cited (Chen *et al.*, 2013; Chen *et al.*, 2014). Briefly, women who are undergoing benign gynecologic procedures are consented to donate a sample of their endometrial biopsies via the Committee on Human Research (CHR) at UCSF (CHR Protocol, catalog number: 10-02786). Each organization should ensure that the appropriate IRB protocols are being utilized.
7. Digestion media (see Recipes)
 - a. Collagenase I [1 gm/(249 U/mg)] (Worthington Biochemical Corporation, catalog number: LS004196)

- b. Hyaluronidase from sheep testes (856 U/mg solid) (Sigma-Aldrich, catalog number: H 2251)

Note: This product has been discontinued. A comparable substitute is Sigma-Aldrich H2126 at equal kU/ml. It is possible to use other comparable products/classes of hyaluronidase as long as the U/mg is comparable and the product has comparable bioactivity.
 - c. HBSS w/ Mg^{2+} and Ca^{2+} , pH ranging 6.7-7.8 (UCSF Cell Culture Facility)
 - d. HBSS w/o Mg^{2+} and Ca^{2+} , pH ranging 6.7-7.8 (UCSF Cell Culture Facility)
 - e. Dulbecco's phosphate buffered saline (pH 7.2) (PBS) (UCSF Cell Culture Facility)
 - f. Penicillin Streptomycin (Pen/Strep, 1 nM 1x working solution) (UCSF Cell Culture Facility)
8. Transfer media (see Recipes)
 - a. Fetal bovine serum (FBS) (Charcoaled/Dextran Stripped, sterile filtered, virus and mycoplasma tested) (Gemin Bio-Products, catalog number: 100-119)
 - b. MCDB-105 medium (powder) with trace elements (Sigma-Aldrich, catalog number: M6395) (see Recipes)
 - c. 1N NaOH, cell culture grade (Sigma-Aldrich, catalog number: S2770)
 - d. ddH₂O
 - e. Pen/Strep
 9. Stromal cell medium (SCM) (see Recipes)
 - a. DMEM without phenol red (Life Technologies, Gibco[®], catalog number: 21063-029)

Note: Currently, it is "Thermo Fisher Scientific, Gibco™, catalog number: 21063-029".
 - b. Sodium pyruvate solution (1 mM working solution) (Sigma-Aldrich, catalog number: S8636)
 - c. MCDB-105
 - d. FBS
 - e. Antibiotic Antimycotic (AB/AM, 1 nM 1x working solution) (UCSF Cell Culture Facility)
 - f. Gentamycin (gent, 0.1 nM 1x working solution) (UCSF Cell Culture Facility)
 10. Cell culture materials and reagents
 - a. BioCoat™ Matrigel[®] Matrix Thin Layer 24 Well Clear Flat Bottom TC-Treated Multiwell Plate (Thin Layer 100 $\mu\text{g}/\text{cm}^2$) (Corning, catalog number: 354605)
 - b. 40 μm sterile cell strainer (fits onto a 50 ml Falcon tube) (BD Biosciences, Falcon[®], catalog number: 352340)

Note: Currently, it is "Corning, catalog number: 352340".

- c. Defined Keratinocyte Serum Free Media (KSFM) (Life Technologies, Gibco[®], catalog number: 10785-012) [comes as part of a kit including the growth supplement (Life Technologies, Gibco[®], catalog number: 10784-015)]
Note: Currently, it is "Thermo Fisher Scientific, Gibco™, catalog number: 21063-029 and 10784-015".
- d. Accutase cell detachment solution (Merck Millipore Corporation, catalog number: SCR005)
- e. Red blood cell lysis buffer (see recipes)
 - i. Ammonium chloride (NH₄Cl) (Sigma-Aldrich, catalog number: 254134)
 - ii. Potassium bicarbonate (Sigma-Aldrich, catalog number: 237205)
 - iii. EDTA (500 mM stock) (Teknova, catalog number: E0306)
- f. PBS
- g. 0.25% Trypsin (UCSF Cell Culture Facility)

Equipment

1. Rotator (Multi-Rotator) (any rotator will do) (Biosan, model: Multi-bio RS-24)
2. Fisherbrand™ Cell Strainers (Thermo Fisher Scientific, catalog number: 22363547)
3. Centrifuge (any appropriate centrifuge will do) (Thermo Fisher Scientific, model: Sorvall Legend RT+ centrifuge)
4. Incubator (Thermo Fisher Scientific, model: Forma 3110 CO₂ Water Jacketed Tissue Culture Incubator)
5. Serological pipettes
6. Sterile Forceps, scalpels, and other surgical tools (Thermo Fisher Scientific)
Note: Stainless steel, fully autoclavable and suitable for gamma radiation sterilization. The exact catalogue numbers for the set currently used are unavailable but these items are similar across vendors.
7. Brightfield microscope (inverted for cell culture)

Procedure

- A. Tissue digestion procedure
 1. Endometrial biopsies should be transferred in a 15 ml Falcon tube in transfer medium and in a chilled carrier (or in the presence of ice packs).
 2. Endometrial tissues will remain stable at 4 °C for 24 h and do not need to be processed immediately.
 3. Prepare a 15 ml Falcon tube with 5-7 ml 1x digestion media.
 4. Transfer the tissue and media into a petri dish. Use forceps and scalpels to gently pull the endometrium away from the myometrium if utilizing a full tissue section. Discard

the myometrium. Endometrial biopsies procured by pipelle endometrial suction curette are easy to dissect and should not require extensive force. Cut the tissues into 1 mm³ pieces.

5. Observe by brightfield microscopy. When viewed with a 50x lens, endometrial blocks will look dark with visible glandular/luminal fragments embedded within. Epithelial fragments appear to have a shiny, worm/pearl-necklace-like morphology while non-epithelial tissue pieces appear as dark shapes. The epithelial fragments will appear as if they are “embedded” in these dark shapes. Single cells are also observable at this magnification.
6. Use a 2 ml-5 ml pipet to pull up the pieces and transfer to a new 15 ml Falcon tube. Centrifuge at 300 $\times g$ for 1-2 min to pellet the endometrial tissues and single cells, discard the supernatant, and resuspend in 5-10 ml 1x digestion media (10 ml for larger, two-three pipelle pass biopsies).
7. Incubate on rotator (10-20 rpm depending on the model) for 1-2 h at 37 °C (a sterile incubator can be used for this). Gently shake the tube manually every 15 min to assist with digestion.
8. The digested material will now comprise mainly of single cells and epithelial fragments (which is composed of luminal epithelial sheets and glandular epithelium). Compared to pre-digestion, they will now appear free floating and not “embedded” in the dark shapes. If non-digested tissue remains, carefully pipette out these fragments by transferring the digested matter into a petri dish. Continue to A9.
9. Pipette the digested matter into a 40 μm cell strainer placed on top of an open 50 ml Falcon tube.
10. The flow-through contains a heterogeneous mix of leukocytes, endometrial stem cells, and stromal fibroblasts.
11. Figure 1 represents a schematic summary of the digestion and separation procedure.

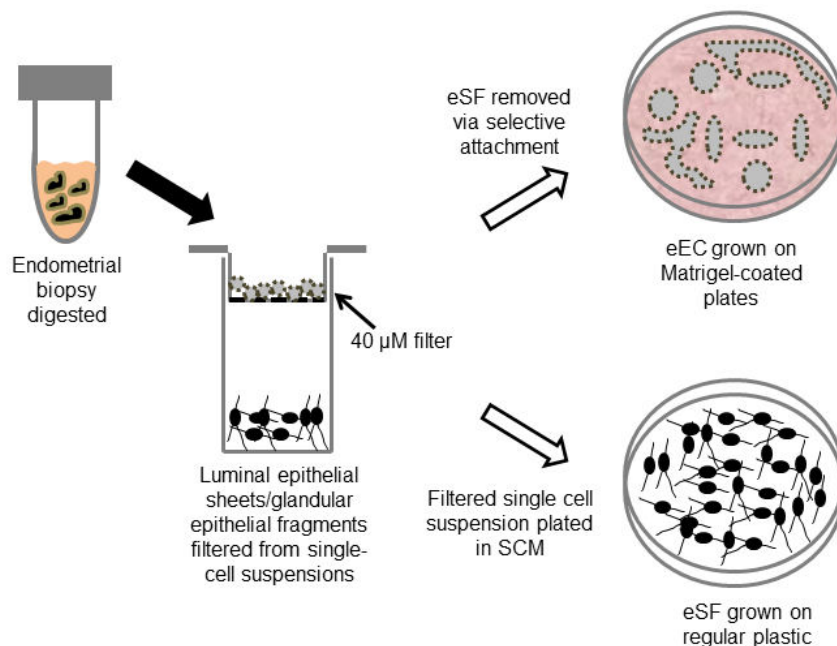


Figure 1. The endometrial tissue digestion and cell plating procedure

B. Culturing of primary eEC

1. Reverse-wash the filter into a petri dish with PBS by turning the filter upside down and using PBS to wash the retained materials off into the dish. The contents should contain luminal and glandular epithelial fragments. Incubate the petri dish with 10 ml of a 1:10 dilution of SCM in PBS. During this process, called selective attachment (Zhang *et al.*, 1995; Kirk and Irwin, 1980; Kirk *et al.*, 1978), eSF will attach to the plastic petri dish in the presence of serum, while epithelial fragments will not attach. Incubate for 1 h at 37 °C. Under 50x magnification, it is possible to identify contaminating tissue pieces, and remove them by gentle aspiration using a pipette.
2. Collect epithelial fragments in a 15 ml Falcon conical tube using serological pipette and spin down at 300 x *g* for 5 min to pellet epithelial fragments. Aspirate the 1:10 diluted SCM and wash the pellet two more times with 10 ml of defined KSFM at 300 x *g* for 5 min to wash out remnants of FBS.
3. (Simultaneously with step B2) Add 500 μl of KSFM to a 24-well Matrigel-coated plate to rehydrate the Matrigel. Incubate at room temperature and remove the medium after 30 min. Add 500 μl of fresh KSFM to the wells.
4. Reconstitute the pellet in KSFM. For the average size endometrial pipelle biopsy (one uterine pass with the pipelle), 3 ml of KSFM is appropriate for the average epithelial yield. Mix the epithelial fragments with a pipette and add 250-500 μl of medium containing epithelial fragments into each well. For the average size pipelle biopsy, there should be enough fragments to plate ~6-12 wells. When viewing the epithelial

fragments, it is optimal to have 5-10 fragments per viewing field per well at 50x magnification for a 24 well plate (Figure 2A).

Note that it is important to mix the fragments after seeding each well since fragments will sediment quickly. Thus, we advise mixing of the bulk solution of fragments before pipetting into each well.

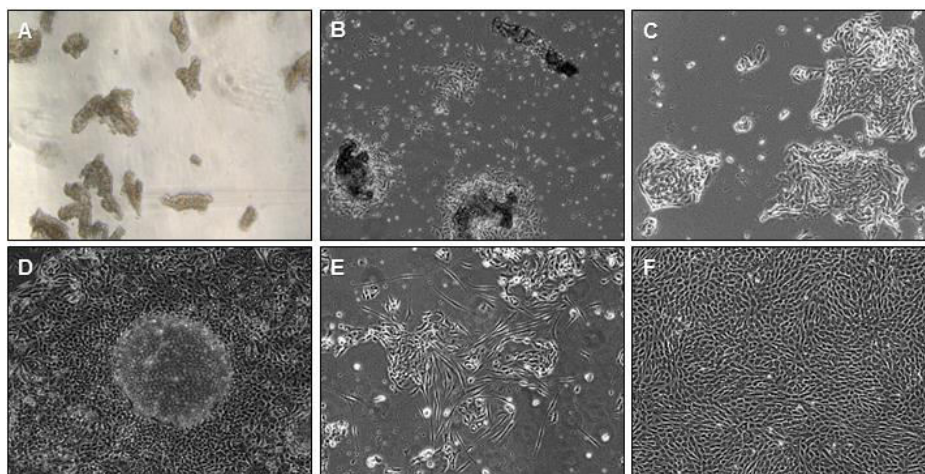


Figure 2. Bright field microscopy of endometrial cell populations. A. Epithelial sheets and fragments; B. Initial attachment of eEC onto Matrigel; C. eEC growing in island-shaped clusters; D. Confluent eEC monolayer with dome-shaped formations; E. eEC culture with eSF overgrowth; F. Confluent eSF. 50x magnification

5. Glands will attach to Matrigel within 24 h (Figure 2B). Attached glands will spread out and grow into island-like clusters (Figure 2C). Eventually these cellular islands will form one major epithelial monolayer. Healthy eEC monolayer will become confluent in 5-10 days, and form dome-like structures (Figure 2D). These domes represent the eEC monolayer folding over each other.
6. The appearance of spindle-like cells likely represents eSF contamination. This type of eSF contamination is common when culturing cells of epithelial origins. Studies report that 1-5% contamination is common (Pierro *et al.*, 2001; Chen *et al.*, 2013), but this contamination does not usually affect the eEC monolayer. eSF in the presence of a low calcium environment such KSMF medium will eventually become non-viable and detach. However, there is the possibility that eSF will propagate even in sub-optimal conditions, and overtake the eEC culture (Figure 2E). These samples are unusable and should be discarded.
7. It is possible to passage the cultured cells once, but not more than that as eEC are reported to have limited expansion potential (Pierro *et al.*, 2001; Chen *et al.*, 2013; Chen *et al.*, 2014). To passage eEC, remove the KSMF and incubate with Accutase (500 μ l for a 24 well plate) at 37 °C for 20-30 min.

Note that trypsin-based reagents may lower eEC viability and should be avoided. After eEC is detached, wash twice in KSFM (for ease of use, it is possible to pool all the wells of cells in Accutase into a 15 ml Falcon tube). Add equivalent volumes of KSFM. Spin again at 300 x g to remove the supernatant. Resuspend the single-cell suspension in KSFM. The volume will vary based on the cell density. Plate 1×10^5 cells into each subsequent 24-well Matrigel-coated plates.

C. Culturing of Primary eSF

1. Centrifuge the filtered single-cell suspension at 300 x g for 5 min (from step A9) to remove digestion media. If necessary, the pellet can be treated with red blood cell lysis buffer for 1-2 min, centrifuged and then washed twice with PBS. More than two treatments with lysis buffer is not recommended.

Note: It is not absolutely necessary to treat with lysis buffer, however blood and mucous may result in poor visualization of eSF cultures.

2. The pellet (containing mostly eSF, but also some leukocytes, stem cells, and endothelial cells) should then be resuspended in SCM then plated directly onto 10 cm cell culture petri dishes. The use of SCM selects for eSF proliferation while disfavoring the survival of non-eSF cells. Plate $2-3 \times 10^5$ eSF into petri dishes, or 5×10^4 cells into a 24-well plate, depending on experimental goals. eSF confluency should be achieved within 5 days (Figure 2F).
3. eSF can be passaged using trypsin-based detaching reagents. Briefly, plated cells are washed once with PBS, and 0.25% trypsin is added for 5 min in the incubator to detach eSF.
4. After detachment with trypsin, neutralize the trypsin with equal volumes of SCM. Centrifuge at 300 x g for 5 min and resuspend in SCM. The volume should be adjusted accordingly based on the viable cell count. eSF are now ready to be plated.
5. eSF can be routinely passaged up to passages 3-4.

Note that it is possible to isolate other cell types from the digested single-cell matter through flow cytometric sorting as previously reported (Chen et al., 2014).

Troubleshooting

1. My eEC fragments are not attaching to Matrigel.
 - a. The Matrigel must be rehydrated before seeding of eEC.
 - b. Increasing the seeding density (fragments per 50x viewing area) may help.
 - c. Over-seeding of eEC fragments may also inhibit growth potential (adding too many fragments will prevent optimal attachment surface area, usually > 10 fragments per viewing area).
 - d. Extend the incubation of eEC on Matrigel to 48 h before washing off non-attached cells.

- e. Patient use of progestin based contraceptives or the presence of chronic inflammatory endometrial disorders may affect eEC growth potential.
2. My eEC culture is becoming contaminated with bacteria/yeast/fungus.
 - a. Adding gent or AB/AM at working concentrations to KSFM can reduce exogenous contamination.
 - b. Increasing the selective attachment incubation time or adding more SCM (1:5 dilution instead of 1:10) to the selective attachment plate since SCM contains antibiotics.
3. My eEC culture has become non-viable due to significant eSF overgrowth.
 - a. If a substantial number of free individual cells are observed immediately prior to pre-plating of eEC, perform an additional filtration step with a 40 μ m cell strainer.
 - b. Increase the selective attachment incubation time.
4. My eSF culture has some residual eEC in the primary culture. Is that ok?
 - a. Some eEC fragments may not be filtered out and remain in the eSF culture. These eEC will undergo attrition in SCM media and become non-viable.
 - b. Additional passaging of eSF to P2 before experimentation will ensure a pure eSF population.

Recipes

1. 2x digestion media

Combine 156 ml of HBSS w/Mg²⁺ and Ca²⁺ to the collagenase I powder
 Add 3.12 ml hyaluronidase (20 kU total)
 Add 3 ml of Pen/Strep (18.8 nM working concentration)
 6.4 mg/ml collagenase type I
 125 U/ml hyaluronidase (final 2x concentration)
 Aliquot 5 ml into 15 ml Falcon tubes and freeze at -20 °C for storage up to a year
 For the 1x working solution, dilute 1:1 in HBSS w/o Mg²⁺ and Ca²⁺
Note: If the tissue is large and a more concentrated digestion is desired, it is not necessary to dilute to 1x. Tissues can be put directly into 10 ml 2x digestion medium. Please also note that PBS or Transfer Media (see below) can substitute for HBSS w/o Mg²⁺ and Ca²⁺ as the diluent.
2. MCDB-105 medium with trace elements

Combine a bottle of MCDB-105 in 1 L of ddH₂O
 Add 15 ml of 1 N NaOH, which should turn the solution pink
 Sterile filter the solution
3. Transfer media

90% MCDB-105
 10% FBS
 1x Pen/Strep

4. Stromal cell medium (SCM)
 - Add 5 ml of sodium pyruvate to 500 ml DMEM
 - 67.5% DMEM
 - 22.5% MCDB-105
 - 10% FBS
 - 1x Antibiotic/Antimycotic (1 nM working concentration) and 1x gent (0.1 nM working concentration)
5. Red blood cell lysis buffer
 - Prepared using ddH₂O
 - 0.155 M NH₄Cl
 - 0.01 M KHCO₃
 - 0.1 mM EDTA (pH 7.3)

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