Video Article

Two Methods for Establishing Primary Human Endometrial Stromal Cells from Hysterectomy Specimens

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Abstract

Many efforts have been devoted to establish *in vitro* cell culture systems. These systems are designed to model a vast number of *in vivo* processes. Cell culture systems arising from human endometrial samples are no exception. Applications range from normal cyclic physiological processes to endometrial pathologies such as gynecological cancers, infectious diseases, and reproductive deficiencies. Here, we provide two methods for establishing primary endometrial stromal cells from surgically resected endometrial hysterectomy specimens. The first method is referred to as "the scraping method" and incorporates mechanical scraping using surgical or razor blades whereas the second method is termed "the trypsin method." This latter method uses the enzymatic activity of trypsin to promote the separation of cells and primary cell outgrowth. We illustrate step-by-step methodology through digital images and microscopy. We also provide examples for validating endometrial stromal cell lines via quantitative real time polymerase chain reactions (qPCR) and immunofluorescence (IF).

Video Link

The video component of this article can be found at <http://www.jove.com/video/51513/>

Introduction

The human uterus corpus is comprised of three layers, the perimetrium (or serosa), the myometrium, and the endometrium. Distinguishing each of these layers is an important step to establish endometrial cell lines. The perimetrium is the outer most layer of the uterus and composed of thin, serous cells. The myometrium is the thick, middle layer of the uterus and comprised of smooth muscle cells. The endometrium is identified as the inner layer of the uterus and includes epithelial and stromal cell populations.

The endometrium is further subdivided into the basalis layer whose stem cell population is hypothesized to repopulate the functionalis layer approximately every 28 days¹. The functionalis layer of the human endometrium undergoes significant biochemical and morphological changes in response to circulating hormones. These hormones are derived from the pituitary gland and the ovaries.

The coordinated production and release of hormones results in a reproductive cycle. The reproductive cycle is designed to prepare the endometrium for potential embryo implantation events. In humans, the reproductive cycle is known as "the menstrual cycle" and divided into three phases – proliferative, secretory, and menstrual. The proliferative phase involves the proliferation of the functionalis endometrial layer whereas the secretory phase is marked by functionalis maturation. Specifically, extracellular alterations, secretions, and cellular differentiation signal a potential implantation. If implantation does not occur before the end of the secretory phase, the functionalis endometrial layer is shed during the menstrual phase. The importance of menstruation and the events that trigger the shedding of the functionalis layer are still being debated. In humans, it has been posed that menstruation is the result of a specific mid-secretory phase differentiation event known as "spontaneous decidualization"². In this manuscript, we provide detailed methodology for both endometrial stromal cell isolation methods, and use a combination of immunofluorescence and digital images to demonstrate efficacy of these approaches. In addition, we apply a commonly used *in vitro* model of spontaneous decidualization to confirm endometrial stromal cell isolation.

Protocol

Hysterectomy specimens used in this manuscript were collected in concordance with a University IRB-approved ethics protocol numbered IRB-HSR #14424.

1. Sample Acquisition from Clinical Source

1. Obtain government and institution-based ethical guidelines and approval documentation before beginning.
2. Conduct all steps in sterile conditions.
3. Preserve patient-derived tissue in media (RPMI or DMEM/High Glucose) in a 50 ml tube at 4 °C if the sample cannot be processed in culture immediately. Samples can be stored in this state for a maximum of 24 hr.
4. Wash tissue sample three times with 1X sterile phosphate buffered saline (PBS) and discard the solution between washes.

2. Preparation of Primary Cell Lines using the Scraping Method

1. Add 4-10 ml of growth media (RPMI or DMEM/High Glucose supplemented with 10% Fetal Bovine Serum and 1% Penicillin-streptomycin) to tissue and add Fungizone (0.25 µg/ml final concentration) for 30 min.
2. Discard the growth media.
3. Wash tissue twice with 1X PBS.
4. Place the tissue on a 6 cm cell culture plate to distinguish between myometrium and endometrium layers (**refer to Figures 2A-2C for further description**). Separate the endometrium.
5. Using a scalpel or razor blade, transect the tissue into small pieces while scratching onto the 6 cm cell culture dish. These scratches facilitate the attachment of the emerging primary endometrial cells. Compared to the trypsin method, more tissue is needed (**see Figure 2D for an approximation**).
6. Gently, add 2 ml of growth media to scratched dish (tissue fragments will be visible and ideally immobilized from the scratching motion).
7. Transfer the plate(s) to a designated cell culture incubator (37 °C and 5% CO₂). Designate a place for primary cell lines, away from other cell culture dishes. Primary cultures are more sensitive to infection and contamination.
8. Examine the cells under a light microscope daily. Small populations of cells should emerge by day two or three from around the sliced tissues (**see Figure 3B**).
9. To maintain cultures, wash gently with 1X PBS and add fresh growth media (2ml) every three days. When proliferation rate increases, additional growth media (3-4 ml) can be added to the 6 cm culture plate(s).
Note: During washing and media changes, pieces of tissue will likely be aspirated. This will not affect growth of adherent cells. Pieces of tissue should be aspirated by the subsequent step (2.10) as new colonies are unlikely to emerge after one week.
10. When the 6 cm plate is approximately 75 - 80% confluent (**see Figure 3B**), passage using 0.05% trypsin. Primary cells cannot be passaged indefinitely - freeze down one plate of cells as soon as two or three plates are maintained. If more passages are required, follow an immortalization protocol (reviewed in Ref³).

3. Preparation of Primary Cell Lines using the Trypsin Method

1. Place a small piece of endometrial tissue (**see Figure 2D for an approximation**) in 2 ml of 0.25% trypsin supplemented with Kanamycin (0.03 mg/ml final concentration).
2. Incubate tissue at 37 °C on rotating platform for 30 min.
3. Briefly vortex the tissue.
4. Centrifuge the sample at 200-400 x g for 2 min and discard the supernatant.
5. Add fresh trypsin and Kanamycin.
6. Incubate the tissue at 37 °C while rotating for an hr.
7. Vortex the tissue for 5-10 sec.
8. Add 2ml of growth media (supplemented with 10% Fetal Bovine Serum and 1% Penicillin-streptomycin) to deactivate the trypsin.
9. Centrifuge cells at 200-400 x g for 2-3 min.
10. Discard the supernatant and add 2 ml of growth media to the pelleted cells.
11. Plate onto a 6 cm cell culture dish. Scraping the plate as in Step 2.5 of **Preparing Primary Cell Lines using the Scraping Method** is not necessary, but enhances the attachment and outgrowth of primary cultures.
12. Monitor cells under a light microscope daily. Cells are usually visible under a light microscope after 24-48 hr (**see Figure 3C**).
13. To maintain the cultures, monitor the cells and change media every 2-3 days as in Step 2.9.
14. To passage the cells, use 0.05% or 0.25% Trypsin when cells reach 75-80% confluency (**see Figure 3C**).

4. Saving Extra Tissue for Analysis (Snap Freezing and Formalin Fixation)

1. Wash sample twice with 1X PBS.
2. Aspirate as much liquid as possible.
3. For Snap Freezing, place endometrial tissue sample in a 1.7 centrifuge tube (**see Figures 2F and 2G**). Place the 1.7 centrifuge tube in liquid nitrogen for 10 sec or until it can be seen that the tissue has frozen down.
Note: Take care not to directly touch liquid nitrogen when preparing samples. Samples can be stored at -80 °C until further processing or analysis.
4. For formalin fixation, cut a small piece of endometrial tissue (**see Figure 2H**), and submerge in 10% buffered zinc formalin. After 24 hr, discard formalin and add 70% ethanol to tissue samples until further processing.

5. Immunofluorescence (IF)

1. Cell fixation
 1. Prepare cells on a culture slide or plate.

2. Gently, wash cells with 1X PBS.
 3. Add pre-chilled (4 °C) Methanol and Acetone (mixed at a 1:1 ratio) for 5 min.
 4. Air dry the slide before proceeding. Slide can be stored at 4 °C for 1-2 weeks if needed.
2. Processing IF
1. Rehydrate cells with 1X PBS for 10 min. For the following Steps 1-6, conduct all washes and incubations on a rotating platform.
 2. Block using 1X PBS supplemented with 1% Bovine Serum Albumin (BSA) and 1% species specific serum (source of 2nd antibody) for 30 min at room temperature (RT).
 3. Incubate in primary antibody (see manufacturers recommendations for antibody dilutions). Refer to **Materials** for specific antibodies. Dilute the primary antibody in fresh blocking buffer as in Step 5.2.2. This incubation can be conducted for at least 2 hr at RT or overnight at 4 °C.
 4. Wash 3 times with 1X PBS for 5 min each.
 5. Incubate in secondary antibody (see manufacturers recommendations for antibody dilutions). Dilute the secondary antibody in fresh blocking buffer as in Step 5.2.2. To prevent photo-bleaching, it is important to conduct this and subsequent steps in the absence of light.
 6. Wash 3 times with 1X PBS for 5 min each.
 7. Thoroughly dry the slides.
 8. Add mounting media and DAPI counterstaining solution.
 9. Apply coverslip and seal the edges using sealant(s). Slides can be stored at 4 °C in the dark for up to 2 weeks.

6. RNA Extraction

1. Pellet 1×10^7 cells by centrifugation. All materials and reagents in this protocol should be RNase free.
2. Lyse cells in 1 ml TRIZOL reagent, and incubate the homogenized samples for 10 min at room temperature to complete dissociation of nucleoprotein complexes.
3. Add 200 μ l of chloroform per 1 ml of TRIZOL. Shake vigorously by hand for 15 sec and incubate for 2-3 min at RT.
4. Centrifuge at maximum speed (15,000 x g) for 15 min at 4 °C. Three layers will result.
5. Transfer the top aqueous phase into a fresh microcentrifuge tube. Add 1 μ l glycogen to increase the RNA yield; however, this step is only necessary when a small amount of RNA is anticipated.
6. Add 0.5 ml of isopropyl alcohol to the aqueous layer, and invert the samples 3-5 times. Incubate samples at RT for 10 min. To increase yield, samples can be placed in -20 °C or -80 °C for 30 min.
7. Centrifuge at maximum speed for 10 min at 4 °C.
8. At this point, a small pellet of RNA should be visible. Discard the supernatant.
9. Wash the RNA with 1 ml of 75% ethanol.
10. Centrifuge at maximum speed for 5 min and discard the supernatant. Samples can be washed once more to rid inorganic contaminants, but this step is not necessary.
11. Briefly, dry the RNA pellet until RNA pellet is dry (usually takes 7-10 min).
Note: One additional step can be used to decrease inorganic matter and decrease drying time. After discarding the supernatant from the ethanol wash, spin samples at maximum speed for an additional minute and aspirate residual liquid. Take care not to aspirate pellet.
12. Dissolve RNA in RNase-free water, depending on the size of the RNA pellet. Volumes typically range from 10-50 μ l.
13. Incubate samples at 55 °C for 10 min, and measure the concentration of RNA.
14. Store samples at -20 °C until further processing.

7. Reverse Transcription

1. Bring the sample RNA (1-3 μ g) to a volume of 9 μ l with H₂O.
2. Heat RNA to 70 °C for 10 min.
3. To generate an AMV master mix, combine the following reagents: 5 μ l of dNTP (working concentration of 50 μ M), 5 μ l of N6 DNA oligos (working concentration of 80 μ M), 5 μ l of 5X AMV buffer, and 1 μ l of AMV. This master mix solution is designed per one sample.
4. Add 16 μ l of the master mix to the heated RNA. The final reaction volume will be 25 μ l reaction.
5. Use the following PCR conditions for reverse transcription: (Stage 1) 42 °C for 90 min, (Stage 2) 95 °C for 5 min, and (Stage 3) 4 °C until further processing.

8. Real Time PCR

1. Conduct PCR reactions using the primers, annealing temperatures, cycle numbers, and reagents found in **Table 1**.
Note: It is ideal to follow manufacturer instructions when using PCR reagents (refer to company and catalog numbers in **Materials**).

9. *In vitro* Decidualization Protocol (Derived from Ref ⁴ and ⁵)

1. Plate endometrial cells.
2. Grow to a confluency of 75-85%.
3. After cells become confluent, wash once with 1X PBS.
4. Quickly, add hormone-free media (Phenol free RPMI supplemented with 5% charcoal strip FBS and 1% Penicillin-streptomycin).
5. After 24 hr, add hormone free media supplemented with medroxyprogesterone acetate (MPA) at a final concentration of 1 μ M and 8-bromoadenosine 3',5'-cyclic monophosphate (cAMP) at a final concentration of 0.5 mM.

6. After at least 48 hr, stop the reaction and save the plate(s) for further processing.

Representative Results

As emphasized in the **Protocol** section, be sure to conduct all methods under government, institutional, and ethical guidelines when handling and preparing human tissue.

Included in this manuscript is an illustration of the general workflow of "the scraping method" (**Figure 1A**) and "the trypsin method" (**Figure 1B**) used to establish primary endometrial cultures. These methods are described in detail in the **Protocol** section (see parts 1. - 3.). Both methods prove successful in the growth of primary endometrial cultures. The advantage to "the scraping method" is the shortened preparation time; however, the time it takes to observe viable cells is usually 2 - 4 times longer compared to "the trypsin method."

Provided are digital images of the initial human tissue received from tissue procurement. The uterine layers can be distinguished by the coarseness of the layer, *i.e.* the myometrium is thick and muscular and the endometrium is thin and more yielding. We have highlighted the thick, smooth muscle layer (myometrium) in white and outlined the endometrial layer of interest in black from two different hysterectomy samples (**Figures 2A and 2B**). In **Figure 2C**, tissues are oriented with the myometrium facing up while the endometrium is positioned down. The thin, perimetrial layer was surgically resected and not highlighted in these images. It is also important to note that the size of the endometrial layer in these digital 2-dimensional images is misrepresented as the actual 3-dimensional layer is very thin compared to the myometrium.

Cutting the appropriate tissue size is important for both "the scraping method" and "the trypsin method." In **Figure 2D**, appropriate sizes are designated for each method above the respective sample. Using one 0.5x0.5x0.5 cm piece for "the trypsin method" [left] is sufficient for establishing a culture. The representative starting tissue for "the scraping method" [right] includes all uterine layers. The final product for "the scraping method" is represented in **Figure 2E**, and it is recommended that at least 1x1x1 cm of endometrial tissue be used to establish viable cultures. The requirement for this much tissue sample is a disadvantage of "the scraping method."

Remaining tissue is often used in numerous ways including protein and RNA analyses. To ensure preservation of tissue quality, it is important to use a "snap freezing" method as in the **Protocol** section (see 4.). This method is also illustrated in **Figure 2F** and **Figure 2G**. Saving extra tissue by formalin fixation is also a common practice of pathologists and researchers. Preparing tissue for formalin fixation is described in the **Protocol** section (see 4.) and illustrated in **Figure 2H**.

Light microscopy is essential for monitoring primary endometrial cultures. Individual endometrial stromal cells should exhibit fibroblast morphology (**Figure 3A**). "The scraping method" typically generates clusters of early emerging cells, primarily along scalpel-induced scratches (**Figure 3B**, Day 2). "The trypsin method" generates both cluster and dispersed cell growth patterns (**Figure 3C**, Day 4). We have included several representative images from the initial plating (day 0) to the first passage of each method (**Figure 3B and 3C**).

Many tissues are defined by their expression of tissue-specific markers such as smooth muscle actin (SMA) for smooth muscle, CD34 for immune stem cells, etc. While gene and protein expression experiments have been conducted for the endometrium⁶⁻¹¹, an endometrial stromal cell specific marker has yet to be discovered. Instead, researchers commonly assess endometrial stroma purity by measuring markers from potential cell contaminants. For example, cytokeratin markers are used to show epithelial populations. However, it is less of a concern, because establishing and maintaining endometrial epithelia are difficult and usually selected against (Ref¹² and **Figure 4A**). If samples were to be obtained during pregnancy, positive expression of HLA-A, -B, -C demonstrates germ, trophoblast cells¹³. On the other hand, like other mesenchymal fibroblasts, endometrial stromal cells express vimentin (CDH1). We demonstrate with immunofluorescence, the expression of vimentin and the absence of cytokeratin and E cadherin (CDH1) in our established endometrial stromal cells (**Figure 4B**).

Finally, we provide functional evidence of primary endometrial culture via an *in vitro* spontaneous decidualization assay. This method was adapted from Ref⁴ and⁵, and involves treatment of cultures with a combination of Medroxyprogesterone acetate (MPA) and 8- bromoadenosine 3',5'-cyclic monophosphate (cAMP) (described in **9. In vitro Decidualization Protocol** and modeled in **Figure 5A**). In the presence of MPA and cAMP, endometrial stromal cells exhibit significantly altered gene expression profiles (reviewed in Ref¹⁴). The frequently published spontaneous decidualization markers are *Prolactin* (*PRL*) and *Insulin-like Growth Factor Binding Protein 1* (*IGFBP1*) (reviewed in Ref¹⁴). The response of these two genes to MPA and cAMP are strong indicators of both spontaneous decidualization and successful establishment of an endometrial stromal culture. We demonstrate this in **Figure 5B**.

Figure 1. Schematic of the two primary endometrial isolation methods. (A) Steps 2.1-2.10 of the scraping method displayed in an organization chart. **(B)** Steps 3.1-3.14 of the trypsin method displayed in an organization chart. [Click here to view larger image.](#)

Figure 2. Processing uterine tissue. (A-C) Clinical uterine samples from two female patients. **A & C** are derived from Patient 1 and **B** is derived from Patient 2. Surgically resected uterine tissue (**left**) and highlighted uterine layers (**right**). (**A & B**) The myometrium is circled in white and the endometrium in black. (**C**) The myometrium is facing the camera. (**D**) Initial representative endometrial sample sizes for both isolation methods are indicated. The trypsin method requires pure endometrium before addition of the trypsin whereas the scraping method can use the entire uterine specimens. (**E**) Example of the processed endometrial sample of the scraping method. (**F & G**) Image of remaining uterine tissue being prepared for Snap Freezing. Shown is a lab-made metal container utilized for this method. (**H**) Formalin fixation of endometrial sample. [Click here to view larger image.](#)

3A.

3B.

3C.

Figure 3. Light microscopy images of primary endometrial cultures. (A) Representative images of endometrial stromal cell cultures taken at various powers and confluency. (B) Representative images of the scraping method (Days 0 - 16) taken of the same culture dish, powered at 10x. **Note:** Visual lines indicate scratches from surgical blade. (C) Representative images of the trypsin method (Days 0 - 16) taken of the same culture dish, powered at 10x. [Click here to view larger image.](#)

Figure 4. Immunofluorescence images of endometrial stromal cells. (A) Immunofluorescence of primary endometrial cultures isolated via the scraping method. Images demonstrate loss of cytokeratin between passage 2 (P2) and passage 5 (P5). Promyelocytic leukemia protein (PML) nuclear protein and DAPI staining were used as controls. **(B)** Images demonstrate positive staining for the mesenchymal marker Vimentin. Images also show negative staining for E cadherin (CDH1) and Cytokeratin. Staining for DAPI and Promyelocytic leukemia protein (PML) were provided as controls. [Click here to view larger image.](#)

5A.

Figure 5. Spontaneous decidualization of two primary endometrial stromal cells. (A) Outline of experimental procedure. **(B)** Upregulation of Prolactin (PRL) and Insulin-like Growth Factor Binding Protein 1 (IGFBP1) gene expression in response to MPA and cAMP. Results were measured via RT-qPCR, and expression was normalized to GAPDH. Significance was calculated using students t-test ($P < 0.001$ *** and $P < 0.0001$ ****). Both cell lines were isolated using the scraping method. [Click here to view larger image.](#)

Primer	Sequence	Annealing Temperature (°C)	Cycles	Assay
Prolactin (PRL) Forward	CATATTGCGATCCTGGAATGAGC	60	40	Sybrgreen
Prolactin (PRL) Reverse	TCCTCAATCTCTACAGCTTTCGA	60	40	Sybrgreen
Insulin-like growth factor binding protein 1 (IGFBP1) Forward	TCCTTTGGGACGCCATCAGGAC	60	40	Sybrgreen
Insulin-like growth factor binding protein 1 (IGFBP1) Reverse	GATGTCTCCTGTGCCTTGCGTA	60	40	Sybrgreen
GAPDH	Unspecified (see reagent list)	60	40	Taqman

Table 1. PCR conditions for decidualization markers.

Discussion

Other groups have described and adapted methodology for the preparation of endometrial stromal cultures, most of which utilize collagenase^{4,12,13,15-18}. In this manuscript, we have provided methodology and evidence for two simplified primary endometrial stromal culture methods, both of which are utilized by our lab for economical reasons and the convenient availability of trypsin and/or a razor blade.

When comparing our two methods, both successfully generate viable primary cultures. Our preferred method is the trypsin method as there is typically a higher yield with a smaller sample size requirement. However, if preparation time is limited, the scraping method requires 30 minutes whereas plating cells using the trypsin method takes more than an hour and a half.

Also noteworthy is that we demonstrate these methods with hysterectomy samples as opposed to endometrial biopsies. Endometrial biopsies are taken without significant intrusion and tend to produce smaller specimens. Still, the methods described in this manuscript (especially the trypsin method) should yield cultures from endometrial biopsies.

There are numerous applications for primary endometrial stromal cells. Comparing endometrial stromal cultures from human versus other mammals may provide clues to the questions that have been debated for centuries about why humans menstruate. There are other unexplored physiology studies that require *in vitro* culture. Of particular interest are the discoveries that provide insights into reproductive cycle events such as the findings that have linked epigenetic molecular events to the functional events of the reproductive cycle^{19,20,21}, and reviewed in Ref¹¹.

Clinicians would benefit greatly from studying endometrial stromal culture and identifying biomarkers in the instances of reproductive disorders and cancer malignancies. Between 2006 and 2010, it was reported that 7.4 million women and their partners used infertility services in the United States²². A significant percent of infertility is due to failure of decidualization²³. Moreover, the relationship between endometrial diseases such as hyperplasia and endometriosis to infertility is only recently being assessed. The capacity to study uterine gynecological cancers through *in vitro* modeling is also invaluable because even though advanced endometrial sarcoma is rare, it can be lethal. By establishing *in vitro* primary cultures, we study the impact of the molecular events associated with the diseases and can generate putative clinical applications.

In conclusion, generating representative and efficacious *in vivo* models for many of these applications is difficult. This makes the development of *in vitro* primary endometrial cultures to study several *in vivo* functions critical. These two endometrial isolation methods, "the scraping method" and "the trypsin method," will help provide the foothold for our understanding of endometrial physiology and pathology.

Disclosures

The authors have nothing to disclosure.

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