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Generation of Multicellular Human Primary Endometrial Organoids

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Cover letter

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June 3, 2019

Kyle Jewhurst, Ph.D.
Science Editor
JoVE

Dear Dr. Jewhurst,

We would like to submit our manuscript entitled, “Generation of multicellular human primary endometrial organoids” for a video publication in *JoVE*.

We have developed a unique organoid model from primary human endometrium that is comprised of both epithelial and stromal cells. This is one of a kind and has not been reported thus far and will be of great interest to the field of endometrial and reproductive biology. This is a significant advancement to the field, providing an innovative model system that can be used to further study the endometrium and I have already received interest in obtaining the protocol to generate these organoids. I know that a video that demonstrates the protocol will be invaluable in making the endometrial organoids.

Just to confirm, we have submitted some of this information in a manuscript to another journal (Journal of clinical endocrinology and metabolism) that describes not only the endometrial organoids but how it responds in a hormonal milieu that is found in polycystic ovarian syndrome. There is one panel in Figure 1 that is the same figure that was submitted to JCEM. Otherwise, all other figures are different from the JCEM submission. This manuscript is currently being revised for resubmission.

Sincerely,

J. Julie Kim, PhD
Professor of OB/GYN

TITLE:

Generation of Multicellular Human Primary Endometrial Organoids

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KEYWORDS:

endometrium, organoids, 3D culture, human primary organoids, hormones, epithelial and stromal cells

SUMMARY:

A protocol to generate human primary endometrial organoids that consist of epithelial and stromal cells and retain characteristics of the native endometrial tissue is presented. This protocol describes methods from uterine tissue acquisition to the histologic processing of endometrial organoids.

ABSTRACT:

The human endometrium is one of the most hormonally responsive tissues in the body and is essential for the establishment of pregnancy. This tissue can also become diseased and cause morbidity and even death. Model systems to study human endometrial biology have been limited to in vitro culture systems of single cell types. In addition, the epithelial cells, one of the major cell types of the endometrium, do not propagate well or retain their physiological traits in culture, and thus our understanding of endometrial biology remains limited. We have generated, for the first time, endometrial organoids that consist of both epithelial and stromal cells of the human endometrium. These organoids do not require any exogenous scaffold materials and specifically organize so that epithelial cells encompass the spheroid-like structure and become polarized with stromal cells in the center that produce and secrete collagen. Estrogen, progesterone and androgen receptors are expressed in the epithelial and stromal cells and treatment with physiological levels of estrogen and testosterone promote the organization of the organoids. This

new model system can be used to study normal endometrial biology and disease in ways that were not possible before.

INTRODUCTION:

The human endometrium lines the uterine cavity and serves as the first contact for the embryo during implantation. The endometrium is comprised of luminal and glandular epithelial cells, supportive stromal fibroblasts, endothelial cells and immune cells. Together, these cell types make up the endometrial tissue which is one of the most responsive tissues to sex steroid hormones[1]. The changes that occur during each menstrual cycle are striking. Appropriate growth and remodeling of the endometrium is required to allow for embryo implantation to occur. Aberrant response to estrogen and progesterone can result in a refractory endometrium that does not allow for successful establishment of pregnancy and can even result in diseases including endometrial neoplasia.

In order to study the hormone responses and essential changes that occur in the endometrium, cells from endometrial tissues excised from patients during surgery or endometrial biopsy have been propagated in cell culture. Endometrial stromal cells preferentially proliferate and propagate readily, and the process of differentiation induced by progesterone can be recapitulated in vitro. As a result, much has been learned during this differentiation process, termed decidualization[2,3]. The other major cell type in the endometrium, the luminal and glandular epithelial cells, however, do not grow well as traditional monolayers, losing polarity, becoming senescent, and having limited proliferative potential. As a result, less is known of their biology and their role in the human endometrium. As many neoplasia originate from the epithelial cells, mechanisms associated with hyperplasia or transformation to cancer cells remain to be fully defined. Furthermore, studies have established that hormone response involves the intimate paracrine actions between the epithelial and stromal cells of the endometrium[4,5].

Recently, a three-dimensional (3D) organoid culture of endometrial epithelial cells was established by two independent groups[6,7], which are the first reports of organoids formed from endometrial tissue. These organoids were comprised of endometrial epithelial cells embedded within a protein matrix (**Table of Materials**) and did not include an important hormonally responsive compartment of the endometrial endometrium, the stromal fibroblasts. As the matrix proteins can vary from lot to lot and can trigger signaling pathways that do not necessarily occur in the tissue, it would be ideal to replace the matrix proteins with components of the endometrium. In the current study, a protocol to generate scaffold-free human endometrial organoids of epithelial and stromal cells of the human endometrium is presented. The presence of stromal cells not only provides the support for epithelial cells but also provides the necessary paracrine actions that have been established to be important for endometrial hormone response[4,8,9].

The new multicellular endometrial organoid offers a model system of the endometrium that is simple to generate and that incorporates both epithelial and stromal cells. These organoids can be used to study long-term hormonal changes and early events of disease such as tumorigenesis due to hormonal imbalance or exogenous insults. The complexity of these organoids could

eventually be expanded to include other cell types, including endothelial and immune cells with possibly myometrial cells to truly mimic human tissue physiology.

PROTOCOL:

Endometrial samples were collected from premenopausal women undergoing routine hysterectomy for benign uterine conditions at Northwestern University Prentice Women's Hospital, according to an Institutional Review Board-approved protocol. Written consent was obtained from all women included in the study.

1. Preparation of agarose molds

1.1. Prior to beginning cell isolation, cast and equilibrate 1.5% agarose micro molds (**Table of Materials**) to house the organoids according to the manufacturer's instructions.

2. Generation of endometrial organoids

2.1. Harvest primary stromal and epithelial cells

2.1.1. In a biosafety cabinet using aseptic technique, prepare enzyme solution (2.5 mg/mL collagenase type I + 0.1 mg/mL DNase I in 10 mL of dispase [500 units]) at 37 °C, and sterile-filter the solution using a 0.2 µm syringe filter into a 15 mL conical tube.

2.1.2. In a biosafety cabinet using aseptic technique, scrape off endometrium from the uterine biopsies and mince tissue under hood into very small pieces with a scalpel.

NOTE: Endometrial tissue that is at least 20 mm x 10 mm x 1 mm is required to generate sufficient numbers of organoids.

2.1.3. Put freshly minced tissue into the enzyme solution in a 15 mL conical tube. Close the cap and wrap the top with a wax film (**Table of Materials**) to prevent contamination.

2.1.4. Put the tube with tissue in a water bath or an incubator at 37 °C for 30 min with gentle shaking (80−100 rpm).

2.1.5. Stack a 100 µm cell strainer on top of a 20 µm cell strainer on a 50 mL conical tube. Filter the solution through the two strainers, and then rinse the 15 mL conical tube with 10 mL of Hank's balanced salt solution (HBSS; **Table of Materials**) and put the wash through the strainer to ensure all cells are collected.

NOTE: The flow-through liquid contains stromal cells and red blood cells (~20 mL total). Epithelial cells are collected on the 20 µm strainer. Chunks of undigested tissue remain on top of the 100 µm strainer. Discard undigested tissue into a biohazard waste container.

2.1.6. Invert the 20 µm cell strainer from step 2.1.5 onto a new 50 mL conical tube and wash the epithelial cells off the strainer with 20 mL of organoid media (**Table of Materials**) supplemented with 1% pen/strep.

2.1.7. Centrifuge the conical tubes from steps 2.1.5 and 2.1.6 at 500 x *g* for 5 min.

2.1.8. With the collected stromal cells, remove the supernatant and resuspend the pellet with 10 mL of red blood cell lysis buffer. Incubate at 37 °C for 10−15 min. Centrifuge the conical tube at 500 x *g* for 5 min, remove the supernatant, and resuspend the pellet with 200−300 µL of organoid media (see step 2.2.2 for further instructions).

2.1.9. With the collected epithelial cells, remove the supernatant and re-suspend with 100 µL of organoid media (see step 2.2.2 for further instructions).

2.2. Seeding cells

2.2.1. After equilibrating the 1.5% agarose molds in wells (1 mold per well) of a 24-well plate, remove organoid media on the outside of the agarose molds and tilt the tissue culture plate so that the medium from the cell seeding chamber of the agarose dishes can also be carefully removed.

2.2.2. View epithelial (from step 2.1.9) and stromal (from step 2.1.8) cell suspensions under the microscope. Add more organoid media to either the epithelial or stromal cell suspensions 100 µL at a time, so that the suspensions are roughly equal in density.

NOTE: Epithelial cells will clump together, and it is not advisable to digest/trypsinize epithelial cells into single cell suspensions.

2.2.3. Combine 1 part of stromal cells with 3 parts of epithelial cells by volume.

2.2.4. Pipette 50 µL (60,000 cells) of the combined cell suspension into the cell seeding chamber of the agarose mold.

2.2.5. Once the agarose molds are filled with cells, carefully add 400 µL of fresh organoid media into the well of the 24-well plate.

NOTE: The medium reaches just below the surface of the agarose dishes and will not cause the cells to float out of the molds. After 2−3 days, cells will settle and start to form organoids.

2.2.6. Change medium every second day with 500 µL of organoid media.

2.2.7. After 2-3 days, change medium to one that is supplemented with 0.1 nM estradiol (E) and 0.8 nM testosterone (T) to promote organization of epithelial and stromal cells.

NOTE: Although organization of epithelial and stromal cells can occur with or without hormones, more organoids will organize in the presence of hormones.

3. Harvesting endometrial organoids for experiments

NOTE: After the experiment is done, endometrial organoids can be processed for histology or RNA analysis. The following steps describe how to process the organoids.

3.1. Histology

3.1.1. Dissolve 1.5% agarose in phosphate-buffered saline (PBS) by boiling. Allow the liquid agarose to cool to approximately 50 °C.

3.1.2. Under a dissecting microscope, tilt the 24-well plate and carefully pipette out the medium from the outside of agarose mold. Carefully pipette out the medium from the interior of the agarose mold to avoid disrupting the organoids.

3.1.3. Under a dissecting microscope, carefully pipette 70-75 µL of warm (50 °C) 1.5% agarose into the chamber of the agarose dish. Be careful not to disturb the organoids.

3.1.4. Let cool at 4 °C for 5 min.

3.1.5. Add 4% paraformaldehyde (PFA) into each well of the 24 well plate and fix the entire sealed agarose mold containing organoids overnight at 4 °C. Then store in 70% ethanol at 4 °C until ready to process for paraffin embedding.

3.2. RNA isolation

3.2.1. Under a dissecting microscope, tilt the 24-well plate and carefully pipette out the medium from the chamber of the agarose mold.

3.2.2. Forcefully pipette 1 mL of fresh organoid medium directly into the agarose mold so the organoids are flushed out of the microwells. Be careful not to create too many bubbles.

3.2.3. Repeat the pipetting again with the same medium from step 3.2.2.

3.2.4. Collect all the medium containing the organoids. Centrifuge at max speed to collect the organoids and proceed to RNA extraction.

REPRESENTATIVE RESULTS:

A schematic of the protocol is depicted in **Figure 1**. Uterine tissue was obtained from surgery after it was examined by pathologists. The endometrial lining was separated from the myometrium by scraping and the endometrial tissue was enzymatically digested to cells as

outlined in the protocol. Epithelial and stromal cells were added into microwells in the agarose molds. After 7 days in culture, organoids were treated with E and T for an additional 7−14 days.

For histological processing, the agarose molds containing endometrial organoids were sealed with agarose followed by fixation with 4% PFA overnight. These molds were processed for standard paraffin embedding, sectioned and stained for histology, immunofluorescence or immunohistochemistry. The hematoxylin and eosin (H&E) staining (**Figure 2A**) revealed a spheroid-like structure with a single layer of cells lining the outside and cells in the center. Cell-specific markers for endometrial epithelial (E-cadherin) and stromal cells (vimentin) revealed epithelial cells surrounding the organoid with stromal cells in the center (**Figure 2B**).

Markers of endometrial physiology confirmed that the endometrial organoids retained certain characteristics of the native tissue. Trichrome staining, which stains collagen blue and cells red, showed the presence of collagen within the center where stromal cells resided, demonstrating active production and secretion of collagen by stromal cells similar to the native tissue (**Figure 3A**). In addition, immunohistochemical staining revealed the presence of estrogen receptors (ER), androgen receptors (AR), and progesterone receptors (PR) in both the epithelial and stromal cells (**Figure 3B**).

FIGURE LEGENDS:

Figure 1: Generation of scaffold-free 3D endometrial organoids from human primary endometrial cells. Uterine tissues were obtained from premenopausal endometrial tissues with benign pathology and the endometrial lining was excised from the tissue piece. Upon enzymatic digestion, endometrial epithelial and stromal cells were seeded into 1.5% agarose molds at a 3:1 ratio by volume and maintained in sex hormone-free medium for up to 7 days. Estradiol (E) and testosterone (T) were added to the 3D cultures to mimic the levels of E and T during the follicular phase of a menstrual cycle[10] and to promote organization of the organoids.

Figure 2: Histology and immunofluorescent staining of cell specific markers on endometrial organoids. Organoids were observed after 14 days of stepwise hormone treatment mimicking the follicular phase of a normal menstrual cycle (0.1 nM E and 0.8 nM T for 7 days, 1 nM E and 0.8 nM T for an additional 6 days, followed by 1 nM E and 1.25 nM T for 1 day). (**A**) H&E staining was done on paraffin embedded organoids. (**B**) Cell specific markers were assessed by immunofluorescent staining of E-cadherin (epithelial, red), vimentin (stromal, green) and 4',6-diamidino-2-phenylindole (DAPI; nucleus, blue) which revealed structural organization of the cells in the organoids. Scale bar = 20 µm.

Figure 3: Endometrial organoids exhibit characteristics of native tissue after 14 days of follicular phase hormone treatment. (**A**) Trichrome staining was done to visualize collagen (blue) and cells (red). Trichrome stain was performed on endometrial tissue (left panel) and endometrial organoids (right panel; scale bars = 20 µm). (**B**) Immunohistochemical staining for ER, AR and PR was done in endometrial tissue (bottom panels; scale bar = 100 µm) and endometrial organoids

(top panels; scale bar = 20 μm). Positive stain is shown in brown and hematoxylin stain solution was added as the counter stain shown in blue.

DISCUSSION:

We have generated human endometrial organoids comprised of epithelial and stromal cells of the endometrium without the use of exogenous scaffold materials. While it has already been shown that primary endometrial epithelial cells can form organoids[6,7], these cells were embedded in a gelatinous matrix of proteins secreted by mouse sarcoma cells (see **Table of Materials**) to help form spheroid-like structures. In addition, endometrial stromal cells were absent. We speculate that the stromal cells in our system, which is an essential supportive component of the endometrial tissue, provided the scaffold for epithelial cells to adhere to and paracrine signaling occurred between cell types. The organization of the epithelial cells around the stromal cells indicated an active interaction between the two cell types which provided the physiologic cues. As hormonal response of the endometrium relies on paracrine interactions between the epithelial and stromal cells[4,8,9], our multicellular organoids provide an alternate, more complex mimic of the native tissue.

The organoids that were generated here were mostly somatic cells, although a small percent of cells was stem-like. We have not yet passaged these organoids for multiple generations and do not know if they would survive and propagate. In addition, endometrial organoids are heterogeneous in nature in that not all organoids contained the same number of epithelial, stromal or stem cells, even those originating from the same tissue. Sizes of the organoids also differed and while most cells formed spheroid like structures, loose cells within each microwell were observed. The presence of E and T appeared to promote organoid formation with the specific organization of epithelial cells lining the outside and stromal cells in the center. We have not tested whether E or T alone would promote organoid formation and what the optimal length of treatment would be. Additional testing of parameters and conditions would be beneficial to generate the ideal endometrial organoids suited for the experiment planned.

Medium is an important component of culturing organoids for promoting growth and survival. From our experience in working with endometrial cells, growth of epithelial cells in culture is the most challenging in contrast to stromal cells which propagate well. Different media were tested, including the organoid media of choice (**Table of Materials**), which is used to generate mammospheres and tumorspheres of breast cancer, which are of epithelial cell origin. Our tests revealed that this medium permitted organoids to maintain their structural integrity and viability over the course of 14−28 day cultures. The effect of this medium on stromal cells, however, needs additional investigation. Despite their survival and production of collagen in the organoid media, the proliferation rate observed in 3D culture was much less than in monolayers. The decreased proliferation may however be due to the 3D structure as well. Given that the organoid media used is commercially available, medium components remain unclear due to its proprietary nature.

In future studies, we envision increasing the complexity of these endometrial organoids to incorporate other cell types found in the endometrium, including endothelial and immune cells.

This is just the beginning of engineering a complete endometrial mimic that can be used to study biology, function, disease, and to test drugs.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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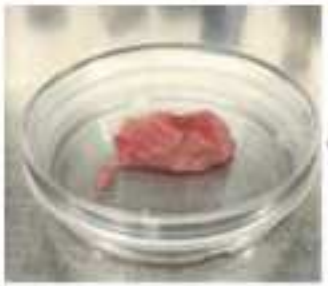
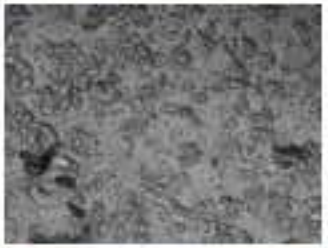
Figure 1



Uterine Tissue



Digested to cells

Cells placed in agarose molds

Cells treated with E and T for 7 days

Figure 2

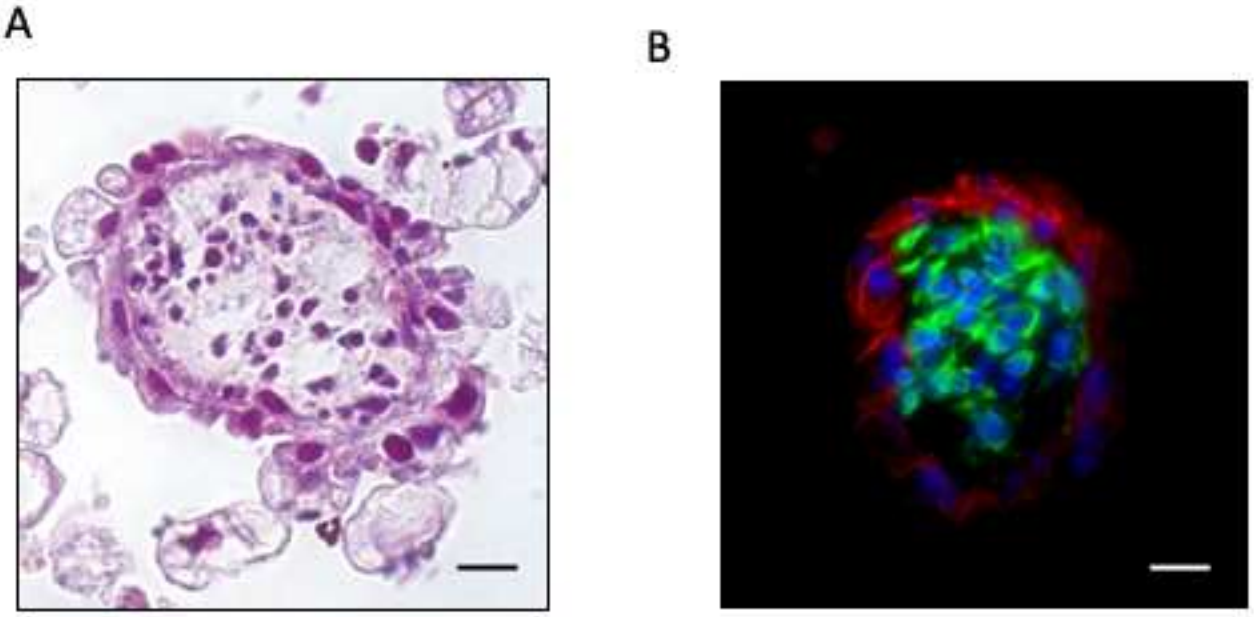
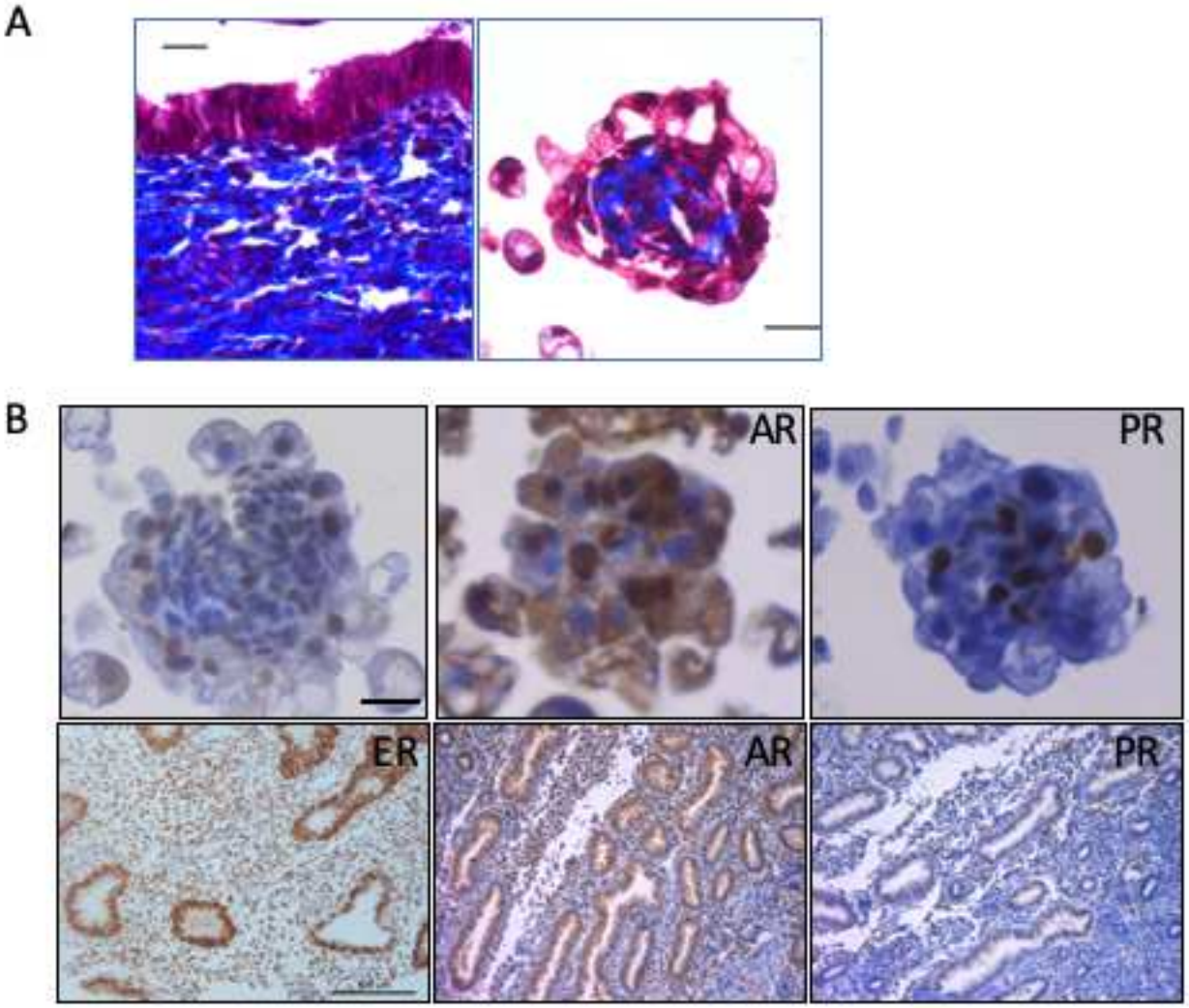


Figure 3



Name of Material/Equipment	Company	Catalog Number
Agarose HS, molecular biology grade	Denville Scientific	CA3510-6
Agarose molds	Sigma-Aldrich	Z764043
Ammonium chloride (NH_4Cl)	Amresco	0621
β-Estradiol	Sigma-Aldrich	E2257
Collagenase, Type II, powder	Thermo Fisher Scientific	17101015
Dispase	Corning	354235
DNase I	Sigma-Aldrich	D4513
EDTA	Fisher Scientific	BP120-1
Eosin Stain	VWR	95057-848
Estrogen Receptor (SP1), rabbit monoclonal antibody	Thermo Fisher Scientific	RM-9101-S
Fluoroshield with DAPI, histology mounting medium	Sigma-Aldrich	F6057
Hank's Balanced Salt Solution (HBSS)	Corning	21-022-CV
Hematoxylin Stain Solution	Thermo Fisher Scientific	3530-32
Heparin solution	STEMCELL Technologies	07980
Hydrocortisone stock solution	STEMCELL Technologies	07925
Organoid media - Mammocult	STEMCELL Technologies	05620
Paraformaldehyde, 16% solution	Electron Microscopy Sciences	15710
Penicillin-Streptomycin	Thermo Fisher Scientific	15140122
Phosphate buffered saline, pH 7.4	Sigma-Aldrich	P3813
Progesterone Receptor, PgR 1294, unconjugated, culture supernatant	Agilent Technologies	M356801-2
protein matrix - Matrigel	BD Biosciences	356231
Purified mouse anti-E-cadherin antibody	BD Biociences	610181
Recombinant anti-vimentin antibody [EPR3776]	Abcam	ab92547
RNA lysis and isolation kit	Zymo Research	R2060
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich	S6014
Testosterone	Sigma-Aldrich	86500

Trichrome Stain	Abcam	ab150686
Wax film - Parafilm	VWR	52858-000

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CORRESPONDING AUTHOR

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Institution:	**Northwestern University**
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Signature:	
Date:	**June 3, 2019**

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After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

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   Proofread.

2. Keywords: Please provide at least 6 keywords or phrases.

   2 more phrases were added

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   Human subjects ethics statement has been included

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1−2 actions and contain 2−3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations

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   This info has been removed.

6. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

   Revised

7. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

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8. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

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9. Please include a scale bar, ideally at the lower right corner, for all microscopic images to provide context to the magnification used. Define the scale in the appropriate figure Legend.

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Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

References have been formatted according to Endnote Jove Style (there is a discrepancy in that the journal title is abbreviated in the endnote style).

12. A minimum of 10 references should be cited in the manuscript. For instance, please include applicable references to previous studies when describing advantages over alternative techniques.

References added.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript describes the establishment of organoids culture from human endometrium. Unlike other reports, this culture system allows for the co culture of epithelial and stroma cells that more reflect the natural juxtaposition of the epithelial cells and stroma cells that will make functional analysis of uterine biology in humans feasible. There are no major concerns .However there are two minor issues that must be addressed in this well written manuscript

Major Concerns:
None

Minor Concerns:
1. What is the starting amount of initial tissue for this procedure. Is there a critical starting weight.
Weight of uterine tissue varies depending on the tissue fragment obtained from Pathology and how much myometrium is present. While the weight of the tissue piece could range anywhere from 1-10 grams in total, the portion that is the endometrium is much less. At least 20mm x 10mm x 1mm of endometrium would be sufficient to generate organoids. This point has been included in the methods.

2 Does stage of the menstrual cycle in which the biopsies taken impact the organoids.

The patient samples that were collected for this study were obtained from both proliferative and secretory phase tissues. In terms of the quality and the number of organoids that form, we did not notice any significant difference. However, a more controlled experiment with more tissue samples from each phase of the cycle would be needed to address this concern.

Reviewer #2:

Manuscript Summary:

The authors describe an exciting protocol for production of primary endometrial organoids from human tissue samples. These results push the field forward by incorporating epithelium and stroma cell types into an organoid structure without the use of an exogenous extracellular matrix. Overall, this method holds promise for increasing our understanding of endometrial physiology by providing a more appropriate in vitro model system for experimentation. The use of testosterone is counterintuitive and requires more explanation, particularly without showing androgen receptor expression in the organoid. Please see additional comments below.

Major Concerns:

The manuscript mentions polarized epithelial cells in the abstract and on line 77, but there is no experimental evidence provided to assess polarity of the epithelial cells. Epithelial cell polarity, and changes in response to steroid hormones, is a key component of normal endometrial physiology and attainment of uterine receptivity to embryo implantation. As such, it is important to verify epithelial cell polarity in the context of this organoid culture system.

We agree with this reviewer that the characteristics of endometrial organoids should retain some characteristics of the native endometrium. We have done a more detailed analysis of the endometrial organoids in the context of response to follicular phase hormones of normal or PCOS conditions in another study that is currently under review (Wiwatpanit et al, 2019). As this JOVE publication is a methods study, we have described the protocol in generating endometrial organoids.

Lines 44 and 159: Please describe how steroid hormones, "promote organization of the organoids." Can you provide images of organoids without E and T treatment? Does E treatment increase proliferation or alter formation of the organoids?

This has been shown in the study currently under review. We have shown that hormones do promote organization in that more organoids have the structural organization than without. We have shown that some cells in the organoids do exhibit marker of proliferation (Ki67) with E + T and this proliferation is increased with the higher T levels for our PCOS hormone treatment condition. To avoid duplication, we did not want to include these studies in this JoVE study but rather we have cited this study (as under review) in the discussion.

Line 78: The hormone responsiveness of the organoids was not determined; expression of nuclear receptors suggests cells are capable of responding to steroid hormones, but doesn't provide direct evidence.

Hormone response to E and T have been demonstrated in the study that is currently under review. In addition, studies looking at progesterone response are in progress.

Line 137: Please describe the number of cells used per agarose petri dish in the representative experiment for reproducibility.

Approximately 60,000 cells (epithelial and stromal cells combined) are plated in each agarose petri dish. This has been added to the protocol.

Lines 155-159: Please explain the rationale for treating the organoids with T? Is androgen receptor expressed the organoid cells or do they have aromatase activity? Additionally, it would

be more physiologically relevant to test the responsiveness of the organoids to progesterone than testosterone. The provided reference states that there is a wide range of T values across the menstrual cycle and there is not a peak observed every patient. Given the levels of circulating T, is there any evidence of a function on the endometrium.

The study was done to test follicular phase hormones on the endometrial organoids. The androgen concentrations are those found in the circulation of cycling women. There are indeed androgen receptors in the organoids and we have now provided a figure. Another study is currently underway to study the progesterone response of the organoids. We have now clarified this in the text.

Minor Concerns:

Lines 119-122: There is no guidance for the suspension volume in the protocol. The next section simply states calculate the cell density/media volume. This doesn't provide adequate instruction for repeating the experiment. The suspension volume will depend on the number of cells collected, but the protocol should list a common range or starting point, i.e. 50 µl is very different from 1 ml.

We have now clarified this procedure. The approximate total cell number to seed one mold is 60,000 cells (epithelial and stromal cells combined). In order to achieve this and keep a ratio of epithelial:stromal cells at 3:1, approximately 7,500 stromal cells are combined with 22,500 epithelial cells. *Note: Epithelial cells will clump together and it is not advisable to digest/trypsinize epithelial cells into single cell suspensions. In order to obtain an approximate 3:1 ratio, stromal cell fraction is diluted with media until it is similar to the epithelial cell density by eye under dissecting microscope. Then 7500 stromal cells (1 part) are combined with 22,500 (3 parts) epithelial cells by volume. Pipette 50 µl of the combined cell suspension into the cell seeding chamber of the agarose mold. (e.g. 33 uL of stromal cells is added to 100 uL of epithelial cells, to make a 3:1 epithelial-to-stromal cell suspension).

Line 157: Missing citation.

It has been added. (*Bui HN, Sluss PM, Blincko S, Knol DL, Blankenstein MA, Heijboer AC. Dynamics of serum testosterone during the menstrual cycle evaluated by daily measurements with an ID-LC-MS/MS method and a 2nd generation automated immunoassay. Steroids. 2013;78(1):96-101.*)

Line 162: This step isn't clear for the reader. The manufacturer's instructions or line 128 could be referenced to indicate that the agarose should be boiled in PBS to dissolve. The agarose likely shouldn't be added to the cells near boiling, so the mixture could be stored at 60ºC before use.

We clarified this step as :
Dissolve 1.5% agarose in PBS by boiling. Allow the liquid agarose to cool to approximately 50°C.

Figures 2 and 3: There are no controls for immunofluorescence or immunohistochemistry shown.

If the reviewer is referring to the controls as antibody testing on native endometrium, we have now included stainings of the native endometrium with ER, PR, and AR. Antibodies for E-cadherin and Vimentin are standard and routinely used. The specific staining of each marker for each cell type supports its specificity for each protein.

Figure 3: There is no description of the counter stain used for the ER and PR immunohistochemistry.

We added the following information to Figure legend 3. Hematoxylin stain solution was added as the counter stain shown in blue.

Reviewer #3:

Manuscript Summary:
This manuscript reports a protocol to generate human primary endometrial organoids that consist of epithelial and stromal cells. Currently, cell culture for human endometrial biology has been limited to in vitro culture systems of single cell types (especially stromal cell culture). They showed that epithelial cells encompass the spheroid-like structure and become polarized with stromal cells in the center that produce and secrete collagen. Both estrogen and progesterone receptors are expressed in the epithelial and stromal cells. These organoids are invaluable to study long term hormonal changes and early events of disease such as tumorigenesis due to hormonal imbalance or exogenous insults. The experiments were well conducted, and the manuscript was well written. I only have some minor suggestions.

Major Concerns:
None noted.

Minor Concerns:

1. Line 75: Clarify weaknesses or limitations of the previous endometrial organoids.

   More detail has been provided.

2. Line 104: Delete "or dry".

   Deleted

3. Line 103-104: "until cell density looks close to the epithelial portion". What does this part mean? How do you measure cell density?
   Line 146: "Mix 3 parts epithelial cell and 1 part stromal cells by volume". It is not clear how many epithelial and stromal cells are used for endometrial organoids. Endometrial epithelial cells are not digested into single cell suspension. Provide detail amount (or measurement method) of epithelial and stromal cells for optimal endometrial organoids.

   This point has been clarified now as described in response to Reviewer 2's concern as well. The approximate total cell number to seed one mold is 60,000 cells (epithelial and stromal cells combined). In order to achieve this and keep a ratio of epithelial:stromal cells at 3:1, approximately 7,500 stromal cells are combined with 22,500 epithelial cells. *Note: Epithelial

cells will clump together and it is not advisable to digest/trypsinize epithelial cells into single cell suspensions. In order to obtain an approximate 3:1 ratio, stromal cell fraction is diluted with media until it is similar to the epithelial cell density by eye under dissecting microscope. Then 7500 stromal cells (1 part) are combined with 22,500 (3 parts) epithelial cells by volume. Pipette 50 µl of the combined cell suspension into the cell seeding chamber of the agarose mold. (e.g. 33 uL of stromal cells is added to 100 uL of epithelial cells, to make a 3:1 epithelial-to-stromal cell suspension).

4. Why do the authors use 0.8 nM testosterone instead of progesterone?

As described, we have tested follicular phase hormones for endometrial organoid generation. The concentration of testosterone during the follicular phase of a cycling woman is approximately 0.8 nM. As progesterone differentiates the endometrium, we did not want this response to prohibit the initial formation of endometrial organoids. We are currently investigating the response of the formed endometrial organoids to progesterone.

6. Figure 2: Add scale bar.

Done