Video Article

Isolation of Mouse Endometrial Epithelial and Stromal Cells for *In Vitro* Decidualization

Katrien De Clercq*[1], Aurélie Hennes*[1], Joris Vriens[1]

[1]Department of Development and Regeneration, Katholieke Universiteit Leuven (KU Leuven)

*These authors contributed equally

Correspondence to: Joris Vriens at joris.vriens@kuleuven.be

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Abstract

Decidualization is a progesterone-dependent differentiation process of endometrial stromal cells and is a prerequisite for successful embryo implantation. Although many efforts have been made to reveal the underlying mechanisms of decidualization, the exact signaling between the epithelial cells that are in contact with the embryo and the underlying stromal cells remains poorly understood. Therefore, studying decidualization in a way that takes both the epithelial and stromal cells into account could improve our knowledge about the molecular details of decidualization. For this purpose, *in vivo* models of artificial decidualization are physiologically the most relevant; however, manipulation of intercellular communication is limited. Currently, *in vitro* cultures of endometrial stromal cells are being used to investigate the modulation of decidualization by several signaling molecules. Conventionally, human or mouse endometrial stromal cells are used. However, the availability of human samples is very often limited. Furthermore, the use of murine tissues is accompanied with variety in the method of culturing. This study presents a validated and standardized method to obtain pure Endometrial Epithelial Cell (EEC) and Stromal Cell (ESC) cultures using adult intact mice treated with estrogen for three consecutive days. The protocol is optimized to improve the yield, viability, and purity of the cells and was further extended in order to study decidualization in a coculture of EEC and ESC. This model may be suitable to exploit the importance of both cell types in decidualization and to evaluate the contribution of significant signaling molecules secreted by EEC or ESC during the intercellular communication.

Video Link

The video component of this article can be found at https://www.jove.com/video/55168/

Introduction

The human endometrium is the inner lining of the uterus and undergoes monthly cycles of breakdown and repair as a preparation for possible pregnancies. It consists of a single layer of epithelial cells lining the uterine lumen and the underlying stroma which varies in thickness according to fluctuations of ovarian hormones estrogen and progesterone. During the luteal phase, the postovulatory progesterone surge will induce differentiation of endometrial stromal cells into larger, round decidual cells, a process called decidualization[1,2]. In human, this phenomenon occurs spontaneously to form the predecidua, but becomes more pronounced upon embryo implantation. A functional consequence of decidualization is that the uterus transiently becomes receptive to embryo implantation. This interval is labeled as the 'window of implantation'. During the early period of embryo implantation, the decidualized endometrial stromal cells surrounding the implanting embryo will provide a barrier to prevent the passage of harmful substances to the embryo[3]. During pregnancy, this decidua will provide nutrients for the developing fetus before placentation has taken place, and will later form the maternal part of the placenta[4].

Ethical and practical considerations limit the design of human studies to investigate the process of decidualization and have prompted the use of animal models. Being a member of the hemochorial placentation group, the endometrium of rodents will also undergo decidualization prior to placentation[5]. In rodents, however, the initiation of the decidualization process depends on the presence of blastocysts in the uterine lumen, indicating that an extra stimulus is necessary to trigger the decidual responses[6]. This implies an intricate communication between the endometrial epithelial cells that have direct contact with the blastocyst, and the underlying stromal cells. Nevertheless, decidualization can be artificially induced using stimuli as mechanical scratching or the instillation of oil in a hormonally primed uterus. Although *in vivo* studies using artificial decidualization models have allowed us to improve our insights in the complex signaling process of decidualization, mechanistic in-depth studies, *e.g.,* investigation of the indispensable communication between epithelial and stromal cells, are limited. Therefore, *in vitro* decidualization studies can be used to manipulate important pathways and study fundamental processes. To this end, primary endometrial stromal cell cultures can be set up from human or rodent endometrium. Employing rodents consents the use of genetically modified animals to investigate the role of a specific protein of interest during the decidualization process. However, immature, prepuberty mice are often used in order to avoid complications of the estrous cycle, while in other cases uteri are collected from all phases of the estrous cycle, which results in a heterogeneity in the cultures. Moreover, the process of decidualization has been studied in different protocols, *e.g.,* by (i) supplementing hormones (estrogen, progesterone or cyclic adenosine monophosphate (cAMP)) to the culturing medium, or by (ii) isolation of decidual cells from

mice at day 4.5 of (pseudo)pregnancy, which demand additional need for plug checking and vasectomized males. These limitations demonstrate the necessity of a standardized method to study *in vitro* decidualization. In this study, a physiological model to examine *in vitro* decidualization starting from adult, non(pseudo)pregnant mice will be presented. In this method, daily injection of 17β-estradiol (E2) will induce proliferation of endometrial cells, resulting in an increased yield of homogenous and pure cell populations. Furthermore, the use of a coculturing system allows the study of the epithelial-stromal crosstalk and the ability to manipulate the process of decidualization on different levels.

Protocol

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All animal experiments for this study were approved by the ethical review committee of the KU Leuven (Belgium) (Project P174/2013). Mice were housed under standard conditions, with *ad libitum* access to food pellets and tap water, and kept under controlled conditions (23 ±1.5 °C, relative humidity 40 - 60%, 12/12 light/dark cycle).

1. Mice

1. Use commercially available mouse strain (*i.e.* C57Bl/6, female 8 - 12 weeks old). Typically, 4 - 5 mice will yield an appropriate amount of cells for further experiments.
2. Inject intact mice subcutaneously with 100 µL of the 17β-estradiol (E2) solution (100 ng/100 µL) for 3 consecutive d prior to the isolation in order to standardize the phase of the estrous cycle of the animals and to induce proliferation of endometrial cells. The need for checking the cycle phase of the mice before starting the protocol is avoidable because of the 3 d' estradiol treatment.

2. Preparations

1. Make a solution of 100 ng/100 µL E2 in arachis oil. For the injections of five mice (as described in 1.2), an amount of 1.5 mL is needed.
    1. Dissolve 1 mg E2 in 1 mL ethanol to have a stock solution of 1 mg/mL.
    2. Dilute this stock solution 1:1,000 in arachis oil.
2. Make 500 mL of Hank's Balanced Salt Solution (HBBS) 1x complemented with 100 U/mL penicillin and 100 µg/mL streptomycin (further referred to as HBSS+).
3. Make 500 mL of filtered Mouse Endometrial Stromal Cell (MESC) medium to culture MESC: Dulbecco's Modified Eagle Medium/ Nutrient Mixture F12 (DMEM/F12) with phenol red, L-glutamine and 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, *N*-(2-Hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) containing 10% FBS, 0.5 µg/mL amphotericin B, and 100 µg/mL gentamicin (further referred to as MESC medium).
4. Make 500 mL of filtered 2% MESC medium to culture MESC during decidualization: DMEM/F12 with phenol red, L-glutamine and HEPES containing 2% FBS, 0.5 µg/mL amphotericin B, and 100 µg/mL gentamicin.
5. Prepare 500 mL of filtered Mouse Endometrial Epithelial Cell (MEEC) medium: DMEM containing 10% FBS, 0.5 µg/mL amphotericin B, 100 µg/mL gentamicin, 25% MCDB-105 medium, and 5 µg/mL insulin (further referred to as MEEC medium).
6. Prepare Poly-L-Lysine (PLL) coated coverslips for MESC culture.
    1. Dissolve 25 mg of PLL in 250 mL of distilled water. Filter the solution using a 0.22 µm filter.
    2. Add sterile coverslips in a 12-well plate. Add 300 µL of dissolved PLL on the coverslips.
    3. Remove the solution after 30 min of incubation. The plates can be stored at 4 °C until further use (up to 1 - 2 months).
7. Prepare a 10 mg/mL stock solution of collagenase type IA and store aliquots of 300 µL at -20 °C. Filter before use.

3. Preparations Required 24 h before Isolation

1. Prepare collagen coated coverslips for MEEC culture.
    1. Prepare a 0.02 M acetic acid solution in distilled water (1 mL per coverslip).
    2. Add 150 µL collagen in 12 mL of 0.02 M acetic acid to obtain a final concentration of 50 µg/mL.
    3. Add sterile coverslips in a 12-well plate.
    4. Add 1 mL of the collagen solution (see 3.1.2).
    5. Incubate O/N (minimum 12 h) at 37 °C.
    During isolation:
    6. Remove the collagen solution off the coverslips by suction and let it air-dry in a sterile environment (in the laminar flow cabinet).
    7. Wash the coverslips twice with Phosphate-buffered Saline (PBS).
2. Prepare a 2.5% pancreatin solution by adding 0.25 g pancreatin in a 15 mL canonical tube containing 10 mL of HBSS+. Shake (200 rpm) the solution at 37 °C for 1 h to increase the solubility. Store at 4 °C.

4. Isolation and Culture of Mouse Endometrial Epithelial Cells (MEEC) and Mouse Endometrial Stromal Cells (MESC)

1. Add 25 mg of trypsin in a 15 mL tube containing 2.5% pancreatin solution (see 3.2) to end up with a final solution containing 0.25% trypsin (further referred to as MESC digestion mix).
2. **Isolation of uteri**
    1. Check the cycle phase of the animals with a vaginal smear examination.

1. Restrain the animal and flush the vagina gently with 50 µL PBS 3 - 5 times.
2. Collect the final flush on a glass slide.
3. Examine the material under a bright field microscope using a 10X or 20X objective.
4. Only use mice that are in the estrus phase. This stage is characterized by the presence of cornified epithelial cells in the vaginal lavage (**Figure 1**).

2. Euthanize the animals using an appropriate method such as cervical dislocation or CO_2-induced narcosis.
3. Spray the carcasses with 70% ethanol in order to generate a sterile environment.
4. Using sterile surgical tools, open the abdomen and push the intestines aside to visualize both uterine horns.
5. Grab the uterine horn at the most distal end under the fallopian tube. Dissect the uterine horns from the fallopian tube and remove adipose and connective tissue. Cut out the horn at the distal end above the uterine body and place it in a 35 mm Petri dish with 3 mL of HBSS+.
    1. Repeat this step for the other horn and for the other animals until all uterine horns are collected.
6. Clean the uterine horn (in HBSS+) further under a stereoscope and remove all residual adipose or connective tissue.
7. Cut the uterine horns open longitudinally to expose the uterine lumen and replace the horn in a new Petri dish with fresh HBSS+.
8. Transfer all uterine horns to the 15 mL tube containing pancreatin and trypsin.
9. Incubate horizontally for 60 min at 4 °C on an orbital shaker (50 rpm).
10. Incubate horizontally for 45 min at 23 °C (RT), without shaking.
11. Incubate horizontally for 15 min at 37 °C (water bath), without shaking.
12. Meanwhile make the MESC digestion mix by dissolving 300 µL of the 1 mg/mL collagenase stock in 2.7 mL of 0.05% Trypsin-Ethylenediaminetetraacetic Acid (EDTA) solution.
    NOTE: From now on, further isolation steps are performed in a sterile environment under a laminar flow cabinet.

3. **Mouse Endometrial Epithelial Cell (MEEC) Culture**
    1. After 2 h of incubation, carefully pour away the supernatant solution.
    2. Transfer the uteri into a Petri dish containing cold MEEC medium and incubate for 5 min in order to inactivate trypsin activity.
    3. Transfer the uteri to a 15 mL tube containing 3 mL of cold HBSS+.
    4. Vortex for 10 s to release the epithelial sheets.
    5. Rinse the uteri in a clean Petri dish with 3 mL HBSS+.
    6. Repeat step 4.3.2 - 4.3.4 to obtain a total of three suspensions containing epithelial sheets.
    7. Transfer the uteri to the MESC digestion mix and incubate for 30 min at 37 °C while shaking (200 rpm) (go to step 4.4 for further isolation of MESC).
    8. Recover the epithelial sheets by pipetting the three cell suspensions gently on a 100 µm nylon mesh in order to remove tissue debris.
    9. Centrifuge the collected cell suspension at 500 x g for 5 min.
    10. Resuspend the pellet in 12 mL of MEEC medium and mix well.
    11. Settle the solution for 5 min in order to separate remaining MESC using gravity sedimentation.
    12. Remove carefully the upper 2 mL by using a 5 mL pipette.
    13. Centrifuge the cell suspension at 500 x g for 5 min.
    14. Resuspend in MEEC medium by gently pipetting up and down.
    15. Plate the cells on collagen-coated coverslips in the desired density for further experiments (**Figure 2a**).
    16. Incubate the cells at 37 °C in a 5% CO_2 incubator for a minimum of 12 h.
        NOTE: For immunostaining or functional experiments like fluorescent calcium imaging, it is advised to plate the cells directly on the coverslips, while seeding in a 6-well plate is recommended when RNA or protein isolation is desired. Isolation of mouse endometrial stromal cells is divided into two rounds as the purity of the cells after only one digestion can be insufficient. The uteri are digested twice for an optimal cell recuperation and purity. In both rounds the technical interventions are identical. Cells collected from both rounds can be kept for further experiments, as desired by the researcher.

4. **Mouse Endometrial Stromal Cell (MESC) Culture: 1st Round**
    1. Before the start of the first digestion round, prepare a second digestion mix by dissolving 300 µL of the 1 mg/mL collagenase stock in 2.7 mL 0.05% Trypsin-EDTA solution. This mix is needed in step 4.4.8.
    2. Prepare three small Petri dishes with cold (4 °C) HBSS+ and 3 x 15 mL tubes with 3 mL of MESC medium (tube X1, X2 and X3).
    3. After 30 min of incubation, shake the uteri-containing trypsin solution gently for 10 s. MESC cells will detach from the uterine tissue and will remain in the trypsin solution.
    4. Transfer the uteri to the first Petri dish containing 3 mL cold (4°C) HBSS+ and rinse well.
    5. Add 3 mL of MESC medium to the tube originally containing the uterine horns (tube in step 4.4.3) to inhibit trypsin activity.
    6. Transfer the uteri from the Petri dish with cold (4 °C) HBSS+ to tube X1 containing 3 mL of MESC medium and shake gently for 10 s.
    7. Repeat steps 4.4.4 (transfer to a Petri dish with cold (4 °C) HBSS+) and 4.4.6 (transfer to tube X2 and X3) twice.
        NOTE: Finally, this protocol will result in 4 cell suspensions: one tube containing the trypsin solution and 3 tubes with MESC medium containing MESC (tubes X1, X2 and X3).
    8. Transfer uteri in the new MESC digestion, as described in step 4.4.1 and incubate for 30 min at 37 °C, vertically.
    9. Collect the stromal cells by passing the four cell suspensions through a 40 µm nylon mesh. Rinse the mesh with an additional 5 mL of MESC.
    10. Centrifuge the cell suspension at 500 x g for 7 min.
    11. Resuspend the pellet in MESC and plate on PLL-coated coverslips for further experiments with the desired density.
    12. Incubate for a minimum of 4 - 6 h or O/N at 37 °C in a 5% CO_2 incubator.

5. **Mouse Endometrial Stromal Cell (MESC) Culture: 2nd Round**
    1. Prepare three small Petri dishes with cold (4 °C) HBSS+ and 3 x 15 mL tubes with 3 mL of MESC medium (Tubes Y1, Y2 and Y3).
    2. Repeat steps 4.4.3 - 4.4.7.

3. Transfer the last cell suspension together with the uteri to a 40 µm nylon mesh.
4. Collect the stromal cells by passing the content of tube Y1, Y2 and Y3 through the nylon mesh. Rinse the mesh with an additional 5 mL of MESC.
5. Centrifuge the cell suspension at 500 x g for 7 min.
6. Resuspend the pellet in MESC medium and plate on PLL-coated coverslips for further experiments (**Figure 2b**).
7. Incubate for a minimum of 4 - 6 h at 37 °C with 5% CO_2.
   NOTE: For immunostaining or functional experiments like fluorescent calcium imaging, it is advised to plate the cells on coverslips, while seeding in a 6-well plate is recommended when RNA or protein isolation is desired.

5. Vimentin/Cytokeratin Double Staining for Validation of Pure MEEC and MESC Cultures

1. Seed the cells on coverslips.
2. Wash 3 x 5 min with PBS containing calcium and magnesium on an orbital shaker (50 rpm).
3. Fix the cells with 4% paraformaldehyde (PFA) (CAUTION!) for 10 min, not on shaker.
4. Wash 3x with PBS without calcium and magnesium on shaker (50 rpm).
5. Permeabilize the cells with 0.2% Triton X-100 for 10 min on a shaker (50 rpm).
6. Wash 3x with PBS on a shaker.
7. Block with 5% goat serum (GS) in PBS for 2 h on a shaker (50 rpm)
8. Incubate cells with monoclonal rabbit anti-human vimentin (1:500) and monoclonal mouse anti-human pancytokeratin (1:1,000) at 4 °C on a shaker (50 rpm). Antibodies are diluted in PBS with 0.5% GS.
9. Wash 3x with PBS on a shaker (50 rpm).
10. Incubate cells with secondary antibodies (1:1,000 in 0.5% GS) Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-mouse IgG on a shaker.
11. Wash 3x with PBS on a shaker.
12. Mount slides with aqueous mounting medium containing DAPI and dry O/N.
13. Seal the slides with nail polish and keep on 4 °C for further use (**Figure 2a, b**).

6. *In Vitro* Decidualization in a Coculture System

NOTE: Four to five animals are required to establish a coculture system in a 24-well plate. Continue with the following protocol in a sterile environment, preferably under a laminar flow cabinet.

1. Prepare the cell culture inserts during the centrifugation and/or incubation steps of the epithelial cell isolation (as described in 4.3) in an additional 24-well plate.
   1. Add 200 - 400 µL of MESC medium into the desired amount of wells in the 24-well plate.
   2. Place the inserts in the prefilled 24-wells using sterile forceps.
2. Resuspend the MEEC pellet (step 4.3.14) in MEEC medium and divide over the inserts (working volume: 150 - 300 µL per insert). Culture the cells at 37 °C in a 5% CO_2 incubator.
3. Seed the stromal cells in another 24-well plate (compatible with the cell culture inserts) after the second round of stromal cell isolation (step 4.5.6). Use a working volume of 400 µL cell suspension per well.
4. Allow the stromal cells to attach for minimum 4 - 6 h at 37 °C in a 5% CO_2 incubator.
5. Transfer the cell culture inserts covered with epithelial cells from the first 24-well plate into the second 24-well plate covered by stromal cells. Use sterilized forceps or touch the inserts only at their hanging foot.
6. Culture the cells at 37 °C in 5% CO_2 incubator for a period of 3 - 5 d until the epithelial cells form a monolayer and the stromal cells achieve 80 - 90% confluency. Additionally, use the Transepithelial Electrical Resistance (TEER) to monitor the epithelial monolayer by use of a Volt/Ohm epithelial meter as described by Grant *et al.*[7]. Values of 800-1,000/well were sufficient to consider the epithelial monolayer confluent.
7. If necessary, replace the medium every 2 - 3 d using glass pipettes or fine pipet tips. Start with the replacement of the medium of the stromal cells (seeded at the bottom), before replacing the medium in the inserts. It is recommended to use preheated cell culture medium at 37 °C.
8. Prepare the decidualization medium to induce *in vitro* decidualization before starting the experiment. Use a working volume of 400 µL per well of stromal cells.
   1. Prepare the following stock solutions and store aliquots at -20 °C.
      1. Prepare 250 µM medroxyprogesterone 17-acetate (MPA) in ethanol.
      2. Prepare 100 mM stock solution 8-Bromoadenosine 3′,5′- cAMP in ultrapure water.
   2. Make the decidual medium containing 1 µM MPA and 0.5 mM cAMP in MESC medium containing 2% FBS.
9. Gently remove the medium from the wells and add the decidual medium onto the stromal cells. If a control condition is needed, use the MESC medium containing 2% FBS.
10. Remove the medium from the cell culture inserts and add 300 µL of MEEC medium onto the cells.
11. Incubate the cells for 5 d in an incubator at 37 °C in 5% CO_2.
12. After the fifth day, assess the extent of decidualization in stromal cells by RT-qPCR (see below).
13. Validate the viability of the epithelial cells using the Resazurin-based viability reagent.
    1. Prepare a 1:10 solution of viability reagent in MEEC medium.
    2. Transfer the cell culture inserts into a new 24-well plate.
    3. Add 150 - 300 µL of the viability reagent - MEEC solution onto the cells.
    4. Incubate at 37 °C in 5% CO_2 for at least 4 - 5 h or overnight and assess cell viability by observing a color shift (blue to purple/pink).

NOTE: Decidualization can also be evaluated using a mouse prolactin-specific ELISA, as preferred by the researcher.

7.Validation of Decidualization by qRT-PCR

NOTE: For validation of the decidualization by qRT-PCR, it is recommended to perform all test-conditions in duplicate in order to receive a sufficient amount of cells for RNA analysis.
Quantitative RT-PCR is performed as described previously in De Clercq *et al.*[8].
In brief:

1. Isolate the total RNA using a commercial RNA isolation kit.
    1. Assess the quantity and quality of the RNA using commercial methods according to manufacturer's protocol, respectively.
2. Synthesize cDNA, according to manufacturer's protocol starting from 1 µg of total RNA.
3. Use a commercial kit according to manufacturer's protocol to carry out the qRT-PCR reaction.
    1. Run all samples in triplicate.
    2. Make use of the commercial TaqMan Master Mix and specific TaqMan probes for the desired housekeeping genes and prolactin (prl8a2) to carry out the reaction.

Representative Results

General Overview of Protocol

Figure 1A illustrates a general overview of the protocol. A daily injection of E2 (100 ng) for three consecutive days was used to synchronize the animals and standardize the phase of the estrous cycle. The estrous cycle can be assessed by vaginal lavages and is divided into the proestrus, estrus, diestrus, and metestrus phase. The cycle phase can be designated according to the ratio of desquamated cells in the lavage, which are subjected to hormonal changes. Increasing levels of estrogen will make the animals evolve to the estrus phase. This phase can easily be detected by the presence of exclusively cornified epithelial cells and the absence of leukocytes (**Figure 1B**). On day 4, uteri of animals that are in estrus phase are collected and MEEC and MESC are isolated (**Figure 1C**). Decidualization can be induced by adding 0.5 mM cAMP and 1 µM MPA to the 2% FBS medium during an incubation period of 5 d.

Quality Control of MEEC and MESC Cultures

The purity of the primary cell cultures can be assessed by the difference in vimentin and cytokeratin expression. Vimentin is an intermediate filament that is expressed in mesenchymal cells, hence in the endometrial stromal cells. Cytokeratin is an intermediate filament found in the cytoskeleton of epithelial tissue. **Figure 2A** shows the mRNA expression level of vimentin and cytokeratin in MEEC and MESC assessed using qRT-PCR. Vimentin levels are high in MESC and low in MEEC (2.2x higher, $p < 0.001$), while cytokeratin levels are high in MEEC and low in MESC (36x higher, $p < 0.001$). A vimentin/cytokeratin double staining was performed and indicated that stromal cells express vimentin whereas epithelial cells express cytokeratin (**Figure 2B, C**). The absence of primary antibody was used as a negative control for the immunostaining (**Figure 2D, E**). These immunohistological stainings show that both endometrial cell cultures (MEEC and MESC) have a purity of up to 90%.

Decidualization in MEEC/MESC Cocultures

After isolation, mouse endometrial and stromal cells were seeded in a co-culture system to mimic the endometrial environment. Cells were cultured until the epithelial cells formed a monolayer in the cell culture insert (TEER values of 800 - 1,000/well), and the stromal cells reached a sub-confluent state. Decidualization was induced in the stromal cells with the application of 0.5 mM cAMP and 1 µM MPA. After five days of incubation, prolactin mRNA levels were assessed in the stromal cells by qRT-PCR as an indication for decidualization (**Figure 3A**). Prolactin levels were highly expressed in cells subjected to the hormones and nondetectable in cells incubated with the control medium ($p < 0.01$).

Since epithelial cells are hard to visualize inside the cell culture inserts, a viability assay was performed immediately after the five-day incubation period (**Figure 3B**). A mix of normal growth medium and a viability reagent was added to the wells and incubated for a period of minimal 8 - 12 h. The color shift from blue to bright pink indicated the presence of viable epithelial cells. Note that the color shift will only occur when viable cells are present.

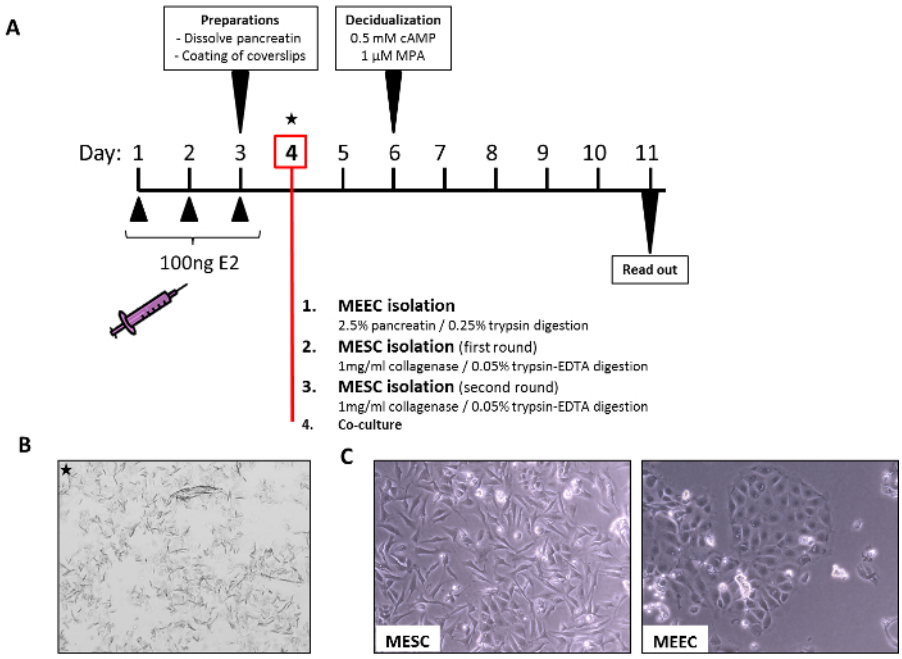

Figure 1: General Overview of the Protocol. (**A**) Scheme of the isolation protocol starting with three consecutive days of estrogen injections. On day 3, the necessary preparations should be prepared. On day 4, estrus phase is evaluated (indicated by star) by performing a vaginal lavage. MESC and MEEC are isolated using different digestion steps. On day 6, or when cells are confluent, decidualization can be induced in the co-culture system by adding cAMP and MPA to the medium and an incubation period of five days (day 6 - 11). (**B**) Representative picture of the estrus phase characterized by the presence of cornified epithelial cells in the vaginal lavage (magnification 10X). (**C**) Representative picture of MESC and MEEC (magnification 20X, scale bar: 100 µm). Please click here to view a larger version of this figure.

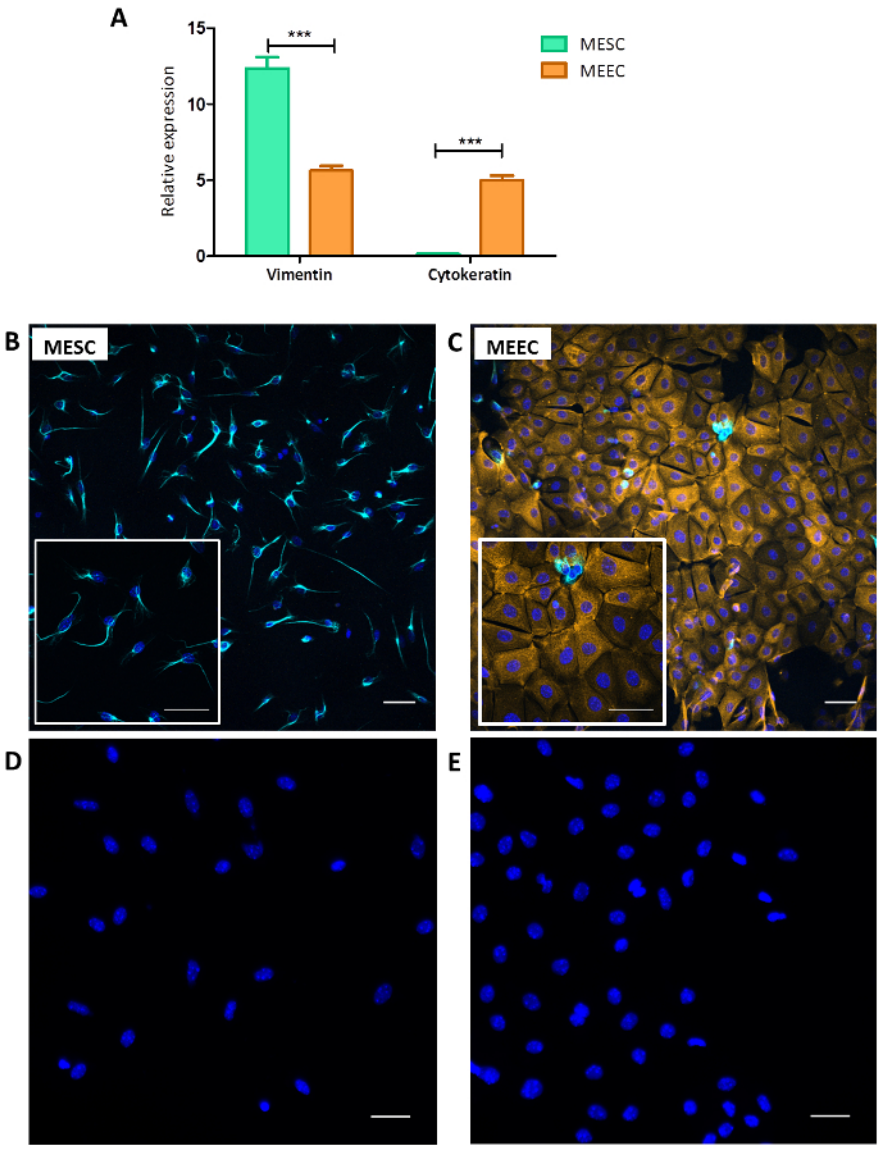

Figure 2: Immunostaining and Quantitative RT-PCR for Vimentin and Cytokeratin in MEEC and MESC. (**A**) Messenger RNA levels of vimentin and cytokeratin expression to indicate the purity of the cultures. RNA levels were relatively quantified to the geometric mean of housekeeping genes ACTB and TBP. Data were shown as mean ± SEM. Vimentin/cytokeratin double staining on MESC cultures (**B**) and MEEC cultures (**C**). Insert in the left shows a 63X magnification view. Negative control with primary antibody omitted for MESC (**D**) and MEEC (**E**) (Scale bar: 50 µm). ***: p <0.001 with Two-way ANOVA with Bonferroni correction for multiple testing. Please click here to view a larger version of this figure.

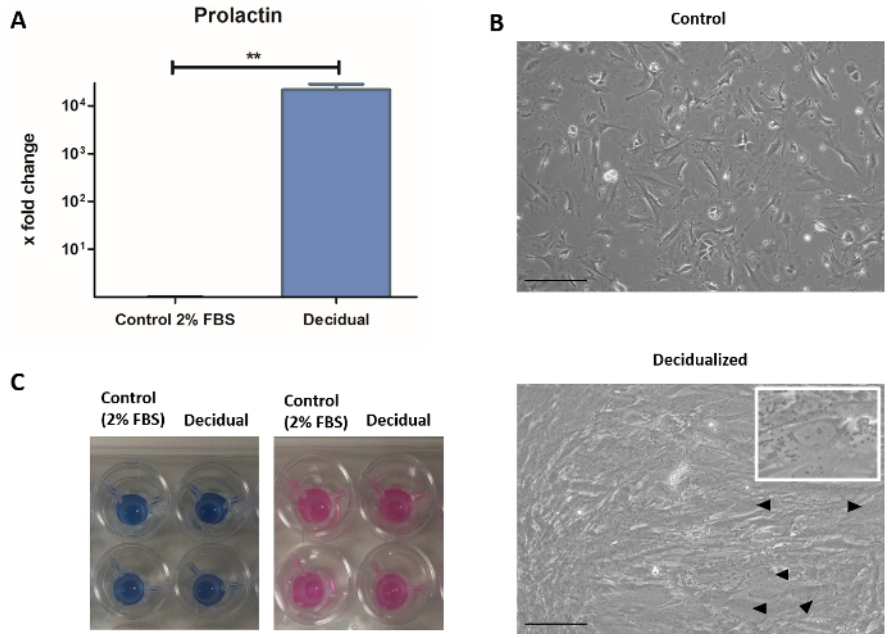

Figure 3: Validation of Decidualization via RT-PCR. (**A**) mRNA levels of prolactin expression in stromal cells to evaluate decidualization. RNA levels were relatively quantified to the geometric mean of housekeeping genes PGK1 and TBP. The fold change in cells cultured in control or decidual medium is shown as mean ±SEM. (**B**) Illustrative pictures of stromal cells before (top) and after (bottom) decidualization stimulus. The insert on the right illustrates a magnification of a decidualized stromal cell. Decidualization is characterized by the presence of multinucleated cells, indicated by black arrows. (**C**) Representative images of the assessment of epithelial cell viability in the insert after the assay. Pictures were taken immediately after the administration of the viability reagent (Prestoblue) (0 h) and the following morning (ON). **: p <0.01 with Mann-Whitney U test. ON: overnight. Scale bar: 100 µm. Please click here to view a larger version of this figure.

Discussion

Decidualization is the progesterone-dependent differentiation of endometrial stromal cells into round secreting decidual cells. In human, this process occurs spontaneously during the luteal phase of the menstrual cycle and is initiated in the stromal cells surrounding the vascular cells to form the predecidua. However, in rodents, the presence of a blastocyst is imperative to induce decidualization. The fact that decidualization only occurs after contact with the endometrial epithelial cells implies that important factors are secreted by the epithelial cells to induce decidualization in the underlying stroma. Although this difference in the initiation of the decidualization process should be acknowledged, the fact that human decidualization becomes more robust upon embryo implantation suggests that similar mechanisms could be involved. *In vitro* cell cultures are often used to study the effect of specific molecules during the process of decidualization. Nevertheless, the availability and amount of human tissue that can be collected is often limited and accompanied by variations, *e.g.,* cycle phase, number of pregnancies and presence of gynecological diseases like endometriosis or adenomyosis. Many of these issues can be prevented by the use of murine tissue that is easily accessible and cycle phase independent. Another strong advantage of using murine tissue is the opportunity to use genetically modified rodents and the possibility to setup a large number of experiments. At the moment, many different isolation protocols have been described in literature with high variability in the age of the animals and the period in the estrus phase at the moment of use.

This study presents a new method for primary endometrial co-cultures of stromal and epithelial cells in which advantage was taken of a standardized estrous cycle by a daily injection of estrogen prior the isolation. Furthermore, estrogen is known to induce proliferation in epithelial and stromal cells which further increases the yield per isolation. The isolation protocol described in this paper was based on Grant *et al.*[7], however, further optimization steps were included to increase the yield, purity, and viability of the different cell cultures. First, HBSS was always supplemented with antibiotics to avoid contamination of the primary culture. During the isolation of MEEC, a temperature adaptation was done (15 min at 37 °C) to increase the harvest of the epithelial cells during the first digestion. Next, an additional step was included to temper enzymatic activity after trypsin-digestion by adding medium containing FBS to inhibit its activity. Furthermore, MEECs were passed through a filter of which the mesh was larger than what is commonly described (100 µm) as they often come in clusters and cell sheets. Moreover, contamination by stromal cells was reduced by performing an additional step in which MEECs and MESCs were separated based on gravity sedimentation. Finally, MEECs were seeded on collagen-coated coverslips to increase the attachment of cells to glass coverslips, as it became clear that attachment to uncoated or PLL-coated glass coverslips was insufficient. During the isolation of the MESCs, collagenase (1 mg/mL) was supplemented to improve digestion. Furthermore, this digestion step was performed in duplicate to avoid contamination of remaining MEECs. All these additional steps resulted in pure and viable cultures of homogenous cell populations.

The purity of the cell population was evaluated with immunostaining and qRT-PCR using vimentin as a stromal marker and cytokeratin as an epithelial marker. Clearly, an opposite expression pattern of vimentin and cytokeratin was observed in MEEC and MESC. However, it can be noticed that the relative mRNA expression of vimentin and cytokeratin is similar in the MEEC cultures. Similar results are published by other research groups, in which epithelial cells in culture acquire vimentin expression[9]. More important is the difference in protein expression between vimentin and cytokeratin as was observed in the immunohistological stainings of the different cell populations. Double immunostaining showed

that EECs were positive for cytokeratin and ESC were positive for vimentin. The use of these markers in immunostaining is an established method and standardly used to indicate the cell types[10].

Although a lot of research has been performed on the decidualization of MESC, it remains possible that the presence of epithelial cells in this model may alter the results of decidualization. Firstly, it has been shown that if MEEC grow to a monolayer in the presence of MESC-conditioned medium or in a co-culture setting, the monolayer has an improved epithelial cell transepithelial electrical resistance (TEER), resulting from factors secreted by MESC[7]. Moreover, Pierro *et al.*[11] provided evidence that epithelial proliferation, influenced by 17β-estradiol, might be mediated by factors secreted from the stromal cells, and alternatively, epithelial cells can release factors that modulate stromal decidualization[12,13]. Overall, it is clear that the epithelial-stromal co-culture environment provides a more physiological situation that allows for paracrine interaction and communication. The method of culturing two different cell types using an insert co-culture system was described earlier[14,15] and was adapted for the use of murine primary cells. By the detection of prolactin mRNA levels as a read-out for decidualization, the described technique has a very reproducible read-out for decidualization available and allows thereby the study of the epithelial-stromal relationship during decidualization. Paracrine signals mediated from MEEC might alter the extent of decidualization in the stromal cells. Moreover, the model allows for specific modulation of the epithelial side without directly affecting the stromal cells. Therefore, this co-culture of MEEC and MESC offers a perfect tool to evaluate the effect of hormones, cytokines, *etc.* on epithelial cells with a read-out on stromal decidualization. Another advantage of this co-culture system is that it allows researchers to investigate the involvement of a specific protein in the decidualization-process in either the epithelial or stromal compartment by using cells isolated from transgenic animals. Furthermore, this technique will be very helpful to investigate blastocyst adhesion to evaluate whether paracrine factors secreted by epithelial cells in the presence of a blastocyst are sufficient to induce decidualization of the underlying stromal cells. An important step in trophoblast invasion correlated to extracellular matrix degradation, which is mediated by the action of proteolytic enzymes. The proposed technique can be applied as an *in vitro* model to investigate the cross-talk among the blastocyst, the epithelial cells and the supporting stroma.

However, at the moment little is known about the origin of the epithelial cells which can be as well luminal as glandular. Therefore, further characterization of the genetic and molecular profile of the epithelial cells is required. In addition, the described method is limited in the available knowledge about the polarization of the epithelial cells. At the moment, the polarization of epithelial cells was not taken into account. However, differences in expression of membrane proteins are described in apical and basal membranes. Inappropriate polarization of the epithelial layer could have an impact on the paracrine interaction between epithelial and stromal cells. However, methods to obtain polarized epithelial cells has been described and could be included in the described technique[15,16].

Altogether, the described protocol proposes a technique to successfully establish primary cell cultures of mouse endometrial epithelial and stromal cells. This technique results in pure cell cultures as confirmed by qRT-PCR and immunostaining of vimentin and cytokeratin. Ultimately, an epithelial and stromal co-culture was introduced that allows for the investigation of paracrine signals mediated by either MEEC and/or MESC in the decidualization process.

Disclosures

The authors have nothing to disclose.

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