Video Article

Isolation of Human Endometrial Stromal Cells for *In Vitro* Decidualization

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Abstract

The differentiation of human endometrial stromal cells (HESC) from fibroblast-like appearance into secretory decidua is a transformation required for embryo implantation into the uterine lining of the maternal womb. Improper decidualization has been established as a root cause for implantation failure and subsequent early embryo miscarriage. Therefore, understanding the molecular mechanisms underlying decidualization is advantageous to improving the rate of successful births. *In vivo* based studies of artificial decidualization are often limiting due to ethical dilemmas associated with human research, as well as translational complications within animal models. As a result, *in vitro* assays through primary cell culture are often utilized to explore the modulation of decidualization via hormones. This study provides a detailed protocol for the isolation of HESC and subsequent artificial decidualization via the supplementation of hormones to the culturing medium. Further, this study provides a well-designed method to knockdown any gene of interest by utilizing lipid-based siRNA transfections. This protocol permits the optimization of culture purity as well as product yield, thereby maximizing the ability to utilize this model as a reliable method to understand the molecular mechanisms underlying decidualization, and the subsequent quantification of secreted agents by decidualized endometrial stromal cells.

Video Link

The video component of this article can be found at https://www.jove.com/video/57684/

Introduction

Between the stages of menarche and menopause, women of reproductive age undergo monthly cycles of hormone-regulated endometrial proliferation, differentiation, and subsequent shedding in preparation for pregnancy in a process known as menstruation[1,2]. Such physical modifications of the human endometrium are necessary for proper embryo implantation into the uterine wall[1]. Alterations of the endometrium, including both morphological and biochemical adaptations, are mediated throughout the menstrual cycle via ovarian steroid hormones estrogen and progesterone (P4)[3,4,5]. Within the proliferative (or follicular) phase, preovulatory estrogen levels increase, initiating endometrium thickening. Following ovulation, the secretory (or luteal) phase promotes a significant rise in P4 concentrations, inducing the morphological transformation of endometrial stromal cells (ESC) from fibroblast-like appearance to rounded, epithelial-like decidual cells in a process known as decidualization[4,6]. Improper decidualization has been established as a root cause for implantation failure and subsequent early embryo miscarriage[4,7,8]. Therefore, understanding the molecular mechanisms underlying decidualization is advantageous to the diagnosis and treatment of early pregnancy loss.

Currently, several methodologies are utilized to explore the underlying effects of decidualization on endometrial stromal cells. *In vivo*, the mouse uterus can be artificially induced for decidualization via mechanical stimulation (*i.e.*,, scratching) or oil injection in a hormonally primed uterus[9]. Distinct from humans, this synthetic stimulation promotes the differentiation of the uterine lumen by providing the appearance of blastocyst presence, a step that is required for the initiation of decidualization in rodents[10,11]. Accordingly, due to the translational complications associated with animal models and the ethical dilemmas surrounding *in vivo* based studies in humans, decidualization based models are most successfully studied *in vitro*.

In this study, subjects are recruited through the placement of advertisements in both local English and Spanish newspapers. Subjects identified as suitable candidates for this study are brought in to meet with the research coordinator, in which a full disclosure of potential risks are discussed. Upon confirmation of a complete understanding of potential risks involved, subjects' consent is attained in both written and verbal forms. Subject consent includes permission to (1) undergo phlebotomy (2) long-term storage of their tissues for future research purposes and (3) agree to the creation of primary cultures from collected tissue specimens. Following consent, subjects are given a form to complete in which permitted self-identification of race/ethnicity and/or the right for nondisclosure. A subsequent visit is scheduled to attain the endometrium biopsy based on the subject's menstrual cycle. Volunteers recruited to this study reflect both the ethnic and racial demographics of the St. Louis metropolitan region as documented by the 2012 Census and did not involve the participation of any vulnerable population including pregnant women, fetuses, embryos, children under 18 years of age, or other vulnerable groups. Eligibility requirements for participation in the biopsy sample collection include (1) being between the ages of 18-45 years (2) having regular menstrual cycles (25-32 days) (3) having no current

pregnancy or use of hormonal/intrauterine device contraceptives for 30 days prior to enrollment (4) having no current vaginal infection or sexually transmitted diseases (5) having no current antibiotic treatments, and (6) having no current abnormal Pap smear.

Within this study, human endometrial stromal cells (HESC) are cultured and artificially induced to undergo *in vitro* decidualization through the supplementation of hormones (estradiol (E2), medroxyprogesterone acetate (MPA), and cyclic adenosine monophosphate (cAMP)) to the medium. In this method, the degree of decidualization is altered based on the total number of days of hormonal treatment. In conjunction with cytoskeletal rearrangement, hormonal supplementation induces biochemical adaptations in which the decidual cells experience secretory-like qualities[2,4]. The expression of hallmark genes, such as prolactin (*PRL*) and insulin-like growth factor binding protein 1 (*IGFBP1*), can be utilized to confirm and quantify the degree of HESC decidualization[5,12,13,14]. Importantly, the viability of this protocol to conduct gene specific knockdown is also demonstrated.

Protocol

All human endometrium biopsies collected for this study were attained from the Washington University in St. Louis, Department of Obstetrics and Gynecology using an Institutional Review Board (IRB) approved written consent form.

1. Preparation

1. Prepare 500 mL of 1x Hank's Balanced Salt Solution (HBSS) by adding 100 U/mL penicillin and 100 µg/mL streptomycin to the media containing bottle (further referenced as HBSS+ medium).
2. Prepare 500 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) with phenol red, L-glutamine, and 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) by adding 1% antibiotic-antimycotic solution (the final concentration is 100 U/mL Penicillin, 100 µg/mL Streptomycin, and 0.25 µg/mL Fungizone) to the media containing bottle (Further referenced as HESC Isolation Media).
3. Prepare 500 mL of DMEM/F12 with phenol red, L-glutamine, and 15 mM HEPES by adding 10% Fetal Bovine Serum (FBS), 1x Na_2HCO_3, and 1% Penicillin (10,000 U/mL) and Streptomycin (10,000 µg/mL) (Pen Strep) to the media containing bottle (Further referenced as HESC Media).
4. Prepare 500 mL of Reduced Serum Medium with L-Glutamine, 2.4 g/L sodium bicarbonate, and HEPES with 2% Charcoal Stripped Fetal Bovine Serum (csFBS) and 1% Pen Strep to the media containing bottle (Further referenced as Decidualization Media).
5. Prepare 500 mL of Phenol red free DMEM/F12 by adding 2% charcoal stripped fetal bovine serum (csFBS) in the media containing bottle (Further referenced as Change Media).
6. Prepare 10 nM estradiol (E2), 1 µM medroxyprogesterone acetate (MPA), and 50 µM cyclic adenosine monophosphate (cAMP) in a 15 mL polypropylene conical tube, and add to a 2 mL per well aliquot of previously prepared decidualization media for *in vitro* decidualization assay (Further referenced as EPC Media).
7. Prepare 50 mL of freezing media with 90% FBS and 10% Dimethyl sulfoxide (DMSO) in 50 mL polypropylene conical tube (Further referenced as Freezing Media).
8. Pre-weigh 25 mg of Collagenase and 5 mg of DNase I in a 50 mL conical polypropylene tube and store at -20°C.

2. Human Endometrium Biopsy Acquisition

1. Obtain human endometrium biopsy samples collected in the proliferative phase (Day 9 - Day 12) of the menstrual cycle from the IRB approved clinic in HBSS.
2. Wash biopsy samples with 1 mL of prewarmed 37 °C HBSS+ medium and gently shake to remove excess blood and mucous.

3. Isolation of Human Endometrial Stromal Cells (HESC)

Note: This isolation procedure is performed in a sterile environment under a biological safety cabinet.

1. Transfer the biopsy with sterilized forceps into a 50 mL conical polypropylene centrifuge tube containing 10 mL fresh HBSS.
2. Centrifuge the biopsy at room temperature at 500 x g for 90 s.
    1. If the cell pellet ruptures, re-spin the biopsy at 2,000 x g for 90 s.
3. Remove the media using a 1 mL pipet and wash with 10 mL of 37 °C prewarmed HESC Isolation Media followed by centrifugation at 500 x g for 90 s.
4. Remove the media using a 1 mL pipet, and vigorously add 3 mL of fresh HESC Isolation Media to dislodge the biopsy.
5. Decant the biopsy into a Petri dish containing 5 mL of HESC Isolation Media.
6. Mince the biopsy into as small pieces as possible using #5 forceps and fine straight stitch scissors for about 20 minutes.
7. Prepare DNase I and Collagenase enzymes by adding 10 mL of HESC Isolation Media to pre-weighed DNase I (0.5 mg/mL) and Collagenase (2.5 mg/mL). Mix with pipet.
8. Pipette cut tissue samples using a 1 mL pipet into a new 50 mL conical polypropylene tube. Wash with 7 mL of HESC Isolation Media and add to the polypropylene tube to acquire the full sample.
9. Centrifuge the sample at room temperature at 800 x g for 2 min. Gently remove the supernatant by pipette.
10. Filter the DNase I and Collagenase solution through 0.2 µm filter attached to 10 mL syringe directly onto pellet.
11. Vortex for 10 s to resuspend pellet.
12. Digest in 37 °C water bath for 1.5 h, vortexing for 10 s thoroughly every 10 min, until tissue is digested.
13. Filter the sample through a 40 µm cell strainer stacked over a 50 mL conical polypropylene tube to remove the epithelial cells and tissue debris.
    NOTE: Stromal cells are in the 50 mL conical polypropylene tube.

14. Centrifuge the 50 mL conical polypropylene tube at 800 x g for 2.5 min at room temperature. Gently remove the supernatant with a 1 mL pipette.
15. Resuspend the pellet in 10 mL of HESC Isolation Media.
16. Centrifuge at 800 x g for 2.5 min at room temperature. Aspirate the supernatant, and then resuspend cells in 6 mL of HESC Media and pipette forward and reverse to mix.
17. Slowly layer on 3 mL of density gradient media to the resuspended cells to separate out remaining blood cells from the stromal cells, being careful not to mix the layers.
18. Centrifuge the 15 mL polypropylene tube at 400 x g for 30 min at room temperature.
19. Collect 5 mL of the supernatant in a new 50 mL polypropylene tube and add an additional 5 mL HESC Media to resuspend the cells.
20. Centrifuge at 800 x g for 2.5 min at room temperature, followed by removal of the supernatant and addition of 6 mL HESC Media. Vortex the sample for 10 s and mix by pipetting forward and reverse 5 times.
21. Aliquot 10 µL of re-suspended cells to a 1.5 mL microcentrifuge tube for cell count.
22. Add 10 µL of 0.4% Trypan Blue stain to cell aliquot and mix with pipet. Load 10 µL of the aliquot and count the cells using a hemocytometer.
23. Plate the cells in an appropriate flask dependent on total cell count in 15 mL HESC Media.
    1. If the total number of cells is less than 1 million, plate all cells in 25-cm flask with 6 mL of HESC Media. If the total number of cells is more than 1 million, plate all cells in 75-cm flask with 15 mL of HESC Media.

24. Culture the cells in a 5% CO_2 incubator at 37 °C for a minimum of 6 h to ensure proper cell adhesion and change the media to HESC Media.
25. Culture the isolated HESC in a 5% CO_2 incubator at 37 °C for a period of 3-5 days until the cells form a monolayer achieving 80 - 90% confluency in a plated flask.
26. Change HESC Media every two days.
    1. Warm HESC Media in a 37 °C water bath for 15-20 min.
    2. Aspirate media from the flask and add fresh HESC Media to the flask (working volume: 8 mL or 16 mL per 25-cm or 75-cm flask respectively).
    3. Place flask back into the 5% CO_2 incubator at 37 °C until 80-90% confluency is achieved.

27. Once HESC become 80 - 90% confluent, transfer cells from 25-cm to 75-cm flask or from one 75-cm flask to three 75-cm flasks.
    NOTE: HESC can be frozen and stored for later usage at this time for future experimentation.

4. Freezing and Thawing HESCs

1. Aspirate media from the 75-cm flask and add 2 mL of trypsin-EDTA.
2. Incubate in a 5% CO_2 incubator for 2-3 min at 37 °C until the cells dislodge from the flask.
3. Add 7 mL of HESC Media and mix well by pipetting forward and reverse.
4. Centrifuge at 800 x g for 2.5 min and aspirate the supernatant.
5. Label freezing tubes with cell line, date, and passage number.
6. Re-suspend the cell pellet in 5 mL of freezing media.
7. Aliquot 1 mL of the re-suspended HESC to each tube and transfer tubes to -80 °C freezer.
8. Transfer tubes to liquid nitrogen within 24 hours.
   NOTE: If planning to thaw cells in immediate future, store frozen cells in -80 °C for one month.
9. For thawing the HESCs preheat HESC Media for 20 min.
10. Add 8 mL of preheated HESC Media to 25-cm flask.
11. Retrieve the freezing tube from the liquid nitrogen tank or -80 °C and submerge the tube in 37 °C water bath for 1-2 min to thaw the cells.
12. Transfer thawed HESC to a 25-cm flask with HESC Media and incubate in a 5% CO_2 incubator overnight at 37 °C.
13. Next day, change the HESC Media and wait for 2-3 days until HESC become confluent and transfer cells to 75-cm flask as detailed below in Protocol 5.
    1. Warm HESC Media in a 37 °C water bath for 15-20 min.
    2. Aspirate media from the flask and add fresh HESC Media to the flask (working volume: 8 mL or 16 mL per 25-cm or 75-cm flask respectively).
    3. Place flask back into the 5% CO_2 incubator at 37 °C until 80-90% confluency is achieved.

5. Human Endometrial Stromal Cell (HESC) Culturing

Note: This Culturing procedure is performed in a sterile environment under a biological safety cabinet.

1. Pre-heat HESC Media at 37°C in a water bath for 20-30 min.
2. Aspirate media from the flask and add 0.25% Trypsin ethylenediaminetetraacetic acid (EDTA) (1 mL for 25-cm flask or 2 mL for 75-cm flask).
3. Incubate in a 5% CO_2 incubator at 37 °C for 2-3 min until the cells dislodge.
4. Gently tap the flask walls to dislodge the remaining cells. Add 7 mL of HESC Media and mix well by pipetting forward and reverse.
5. Pipet the cell mixture into a 50 mL conical polypropylene tube and centrifuge at 800 x g for 2.5 min at room temperature.
6. Aspirate the supernatant and re-suspend the cell pellet in HESC Media.
    1. Add 15 mL for 25-cm flask or 45mL for 75-cm flask (15 mL each).

7. Add 5 mL or 15 mL cell suspension to each 25-cm or 75-cm flask respectively.
8. Culture the isolated HESC in a 5% CO_2 incubator at 37 °C for a period of 3-5 days until the cells form a monolayer achieving 80 - 90% confluency in a plated flask.
9. Change HESC Media every two days.
    1. Warm HESC Media in a 37 °C water bath for 15-20 min.

2. Aspirate media from the flask and add fresh HESC Media to the flask (working volume: 8 mL or 16 mL per 25-cm or 75-cm flask respectively).
3. Place flask back into the 5% CO_2 incubator at 37 °C until 80-90% confluency is achieved.

6. Human Endometrial Stromal Cell (HESC) Plating and siRNA Transfection

Note: This transfection procedure is performed in a sterile environment under a biological safety cabinet.

1. Aspirate HESC Media from the 75-cm flask and add 2 mL of 0.25% trypsin-EDTA solution.
2. Incubate in 5% CO_2 incubator at 37 °C for 2-3 min until the cells dislodge.
3. Once cells are dislodged from the flask, add 7 mL of HESC Media and mix gently by pipetting forward and reverse 5 times.
4. Pipet the cell mixture into a 50 mL conical polypropylene tube and centrifuge at 800 x g for 2.5 min at room temperature. Aspirate the supernatant and re-suspend the cell pellet in 10 mL of HESC Media.
   NOTE: If plating cells from more than one flask, simply increase the HESC Media volume.
5. Count cells using a Hemocytometer.
   1. Aliquot 10 µL of re-suspended cells into a 1.5 mL microcentrifuge tube.
   2. Add 10 µL of 0.4% Trypan Blue stain to cell aliquot and mix by pipetting forward and reverse.
6. Plate 0.8 x 10^5 cells per well in 6-well plates. After two days, cells should be 60-70% confluent for optimal transfection.
7. For each siRNA transfection sample, prepare complexes using siRNA (60 nanomoles) to transfection reagent.
8. Dilute 5 µL of transfection reagent in 150 µL of reduced serum free media and incubate for 5 min.
9. Dilute 60 nanomoles of siRNA in 100 µL of reduced serum free media and incubate for 5 min.
10. After 5 minutes, combine diluted siRNA with diluted transfection reagent solution and mix gently by pipetting forward and reverse 5 times.
    NOTE: If transfecting one or more siRNA in multiple wells, prepare master mix in 10 mL polystyrene tubes.
11. Incubate siRNA-transfection reagent complexes for 5 min at room temperature.
12. During this incubation, add 1 mL of Change Media to each well.
13. After 5 min of incubation, centrifuge tubes at 500 x g for 2 min to collect solution and add the 250 µL of siRNA-transfection reagent complexes to each well containing cells and media.
14. Mix by gently rocking and incubate the cells at 37°C in a 5% CO_2 incubator for 5 h.
15. After 5 h, change the media to HESC Media.
16. Incubate the cells at 37 °C in a 5% CO_2 incubator for 2 days in preparation for an *in vitro* decidualization assay.

7. *In Vitro* Decidualization Assay

Note: This assay is performed in a sterile environment under a biological safety cabinet.

1. Prepare the EPC media prior to initiating the *in vitro* decidualization treatment as described in Camden *et al.*[3] (See 1.6).
2. Aspirate the media from the post-48 hour transfected line of HESC incubated from step 6.16.
3. Add 2 mL of EPC Media into each well.
   Note: As a control, leave one set of cells untreated with EPC Media. Instead, add 2 mL of HESC Media to the control wells.
4. Incubate the cells in 5% CO_2 incubator at 37 °C for 6 days.
   1. Change the EPC Media every 48 hours.
      1. Warm and prepare EPC Media in a 37 °C water bath for 15-20 min.
      2. Aspirate media from the well and add fresh EPC Media.
      3. Place the plate back into the 5% CO_2 incubator at 37 °C.
5. After the sixth day, analyze the extent of stromal cell decidualization via ELISA and qRT-PCR (as described below).

8. Validation of *In Vitro* Decidualization utilizing Quantitative Real Time PCR (qRT-PCR)

Note: qRT-PCR is completed as previously described in Kommagani *et al*[10].

1. Isolate the total RNA from HESC utilizing the protocol from a commercially available RNA isolation kit.
   1. Analyze the RNA quantity and purity (A_{260}/A_{280}) using a NanoDrop or similar methodology as described in Garcia-Elias *et al*[15].
2. Synthesize cDNA from 1 µg of total RNA through a commercially available reverse transcription kit protocol.
3. Run a qRT-PCR reaction as described in a commercially available qRT-PCR kit protocol.
   1. Perform the reaction using a commercially available Real-Time PCR Master Mix and gene specific probes along with internal system controls (*18s*, *PRL*, and *IGFBP1*).

9. Validation of *In Vitro* Decidualization utilizing ELISA

1. Quantify the secreted Prolactin levels from the tissue culture media utilizing a commercially available Prolactin ELISA kit.

10. Validation of *In Vitro* Decidualization utilizing Phalloidin staining.

1. Insert sterile coverslips into each well of a 12 well plate.
2. Repeat steps 6.1 - 6.5.
3. Plate 0.8 x 10^5 cells per well of a 12 well plate.
4. Incubate the cells in 5% CO_2 incubator at 37 °C for 2 days.
5. Aspirate the media.
6. Repeat steps 7.3 - 7.4.
7. Fix the cells in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at room temperature for 30 min.
8. Aspirate the fixation solution and wash 3 times with 1 mL of PBS for 5 min each.
9. Add 0.1% non-ionic surfactant Polyethylene glycol tert-octyl phenyl ether in PBS for 5 min to the fixed cells to increase permeability.
10. Wash 3 times with 1 mL of PBS for 5 min each.
11. Add 100 µL of phalloidin solution per coverslip and incubate at room temperature for 90 min.
    1. Prepare 1:100 dilution in PBS from using a phalloidin stock solution in 1 unit per 5 µL.
12. Wash 3 times with 1 mL of PBS for 5 min each.
13. Mount the slides with mounting medium containing 4',6-Diamidino-2-Phenylindole (DAPI).
14. Observe the cells utilizing a confocal microscope.

Representative Results

Decidualization in HESC Culture

Following isolation, human endometrial stromal cells were cultured to a monolayer formation with 80-90% confluency and induced for *in vitro* decidualization by treating with 10 nM E2, 1 µM MPA, and 50 µM cAMP (EPC). Morphological shifts associated with *in vitro* decidualization were visualized in **Figure 1A**. Upon decidualization, HESCs undergo cytoskeletal rearrangement from elongated, fibroblastic cells to rounded epithelioid cells. Thus, phalloidin staining was performed to confirm the cytoskeletal reorganization during HESC decidualization. Phalloidin is a highly selective bicyclic peptide which is utilized to stain for actin filaments, thereby visualizing the cytoskeletal rearrangement consistent with *in vitro* decidualization (**Figure 1B**).

At six days of post EPC treatments, *PRL* and *IGFBP1* mRNA levels were assessed via qRT-PCR to confirm the degree of decidualization (**Figure 2A**). *PRL* and *IGFBP1* levels were significantly increased compared to the HESC treated with control vehicle ($p<0.001$). Biochemical changes associated with decidualization were also assessed through the quantification of secreted PRL levels from the culturing medium (**Figure 2B**). Consistent with transcript levels, PRL levels were highly elevated in the EPC cultured HESC compared to the control ($p<0.001$).

The effect of specific gene abrogation on the cellular and molecular changes of HESCs decidualization were assessed using *SRC-2* as candidate gene (**Figure 3**). Following 48 hours of transfection with control or *SRC-2* siRNA, HESC were cultured in for 6 days in EPC media. Control siRNA transfected HESC demonstrated cellular and molecular changes consistent with proper decidualization, including a cobblestone morphological change and increased transcript levels of decidualization markers *PRL* and *IGFBP1*. As expected, with *SRC-2* siRNA knockdown, HESC remained fibroblastic in appearance as compared to the control siRNA transfected HESC (**Figure 3A**). Consistent with this cellular change, *PRL* and *IGFBP1* transcript levels were significantly decreased within *SRC-2* siRNA transfected HESC, indicating a derailment in decidualization programing with *SRC-2* knockdown (***$p < 0.001$). *SRC-2* transcript levels were significantly decreased in *SRC-2* siRNA transfected HESC when compared to control siRNA, indicating an efficient gene silencing with our method (***$p < 0.001$) (**Figure 3B**).

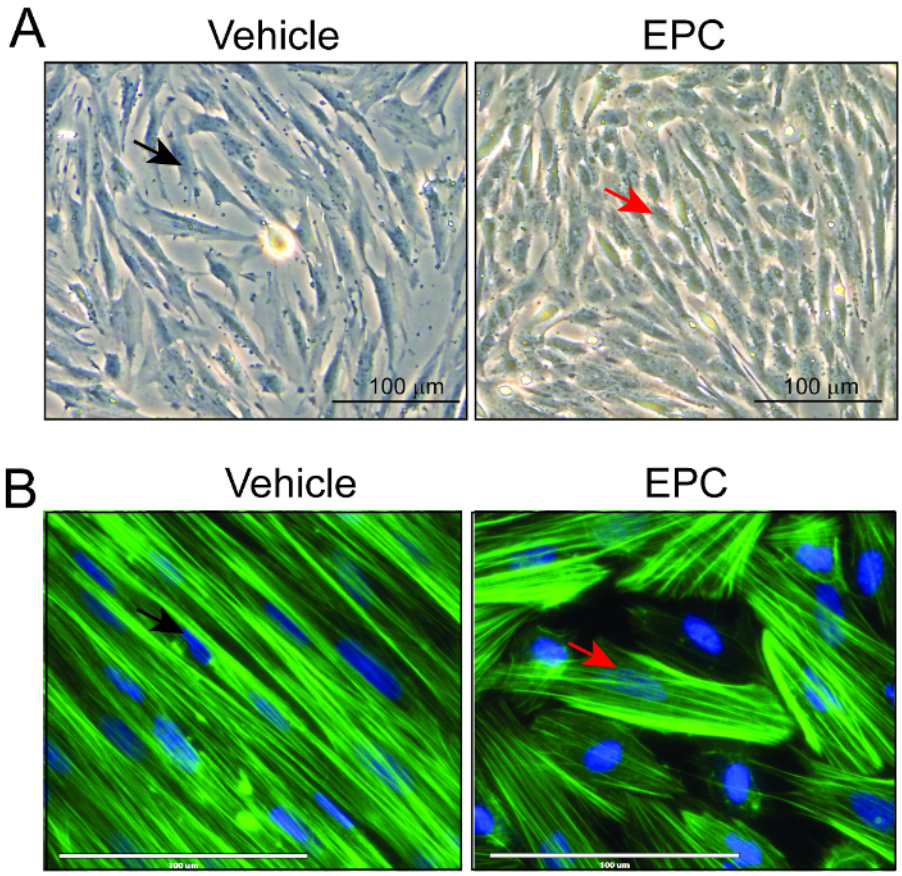

Figure 1: Cellular changes of Human endometrial stromal cells during *in vitro* decidualization. HESCs were isolated and cultured for six days in media containing either vehicle or E2, MPA and cAMP (EPC). A) Cell images illustrating the changes in cellular morphology from fibroblastic to epithelioid cells. B) Phalloidin stained images of HESC illustrating the cytoskeletal changes (in green) associated with *in vitro* decidualization, where blue represents the nucleus (DAPI). The red arrow represents decidualized cells. The black arrow shows fibroblastic cells. Scale bar: 100 µm. Please click here to view a larger version of this figure.

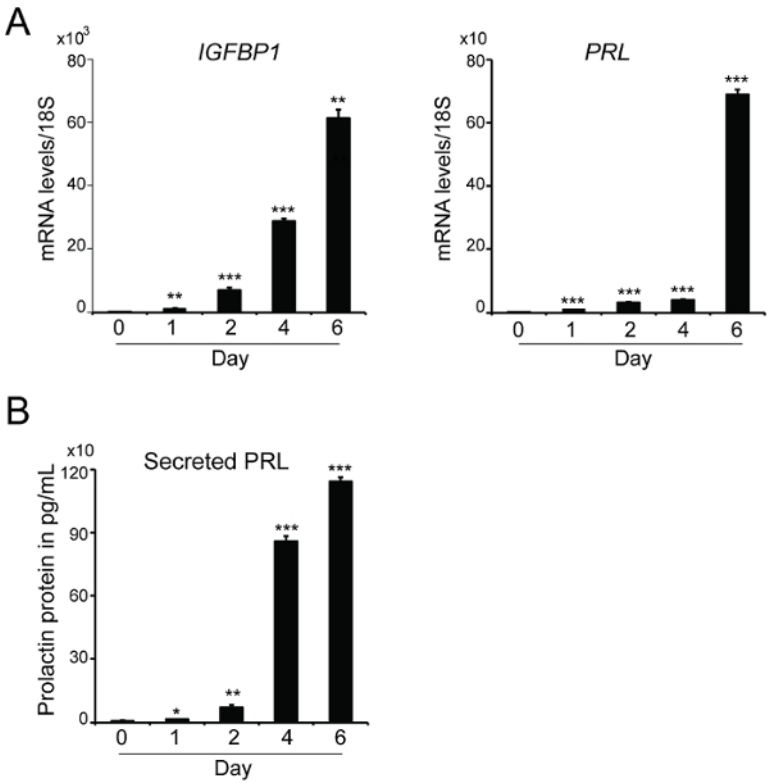

Figure 2: Molecular changes in Human endometrial stromal cells during *in vitro* decidualization. A) Total RNA was isolated from HSECs cultured for six days in media containing either vehicle or E2, MPA and cAMP (EPC) and subjected to qRT-PCR analysis to detect *PRL* and *IGFBP1* transcript levels. B) Levels of PRL secreted into the culture media were measured using an ELISA based assay from HESC cultured for six days in either vehicle to EPC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Please click here to view a larger version of this figure.

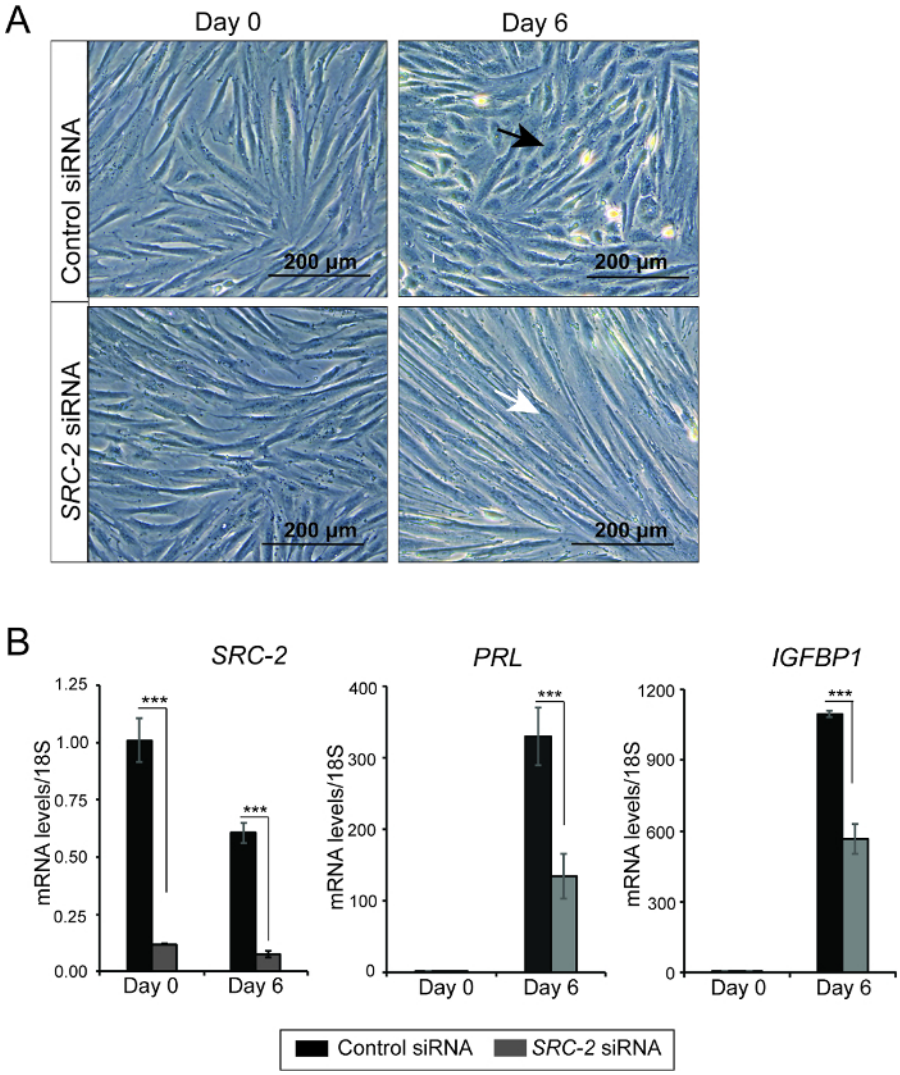

Figure 3: Knockdown of *SRC-2* in Human endometrial stromal cells affects *in vitro* decidualization. A) Morphological changes in siRNA transfected HESC following 6 days of culture with EPC media (top panels: control siRNA at time points Day 0 and Day 6, bottom panels: *SRC-2* siRNA at time points Day 0 and Day 6). B) Transcript levels of *SRC-2*, *PRL*, and *IGFBP1* on Day 0 and Day 6 of EPC treated HESC transfected with control or *SRC-2* siRNA. The black arrow represents decidualized cells. The white arrow shows fibroblastic cells. Scale bar: 200 µm. *** $p < 0.001$. Please click here to view a larger version of this figure.

Discussion

The female reproductive menstrual cycle is characterized by a rise in progesterone levels throughout the luteal phase, thereby inducing the decidualization of ESC into round, epithelial-like secretory cells[3,8]. The initiation of decidualization is species dependent. In humans, decidualization occurs spontaneously upon the rise of progesterone concentration, whereas mice require blastocyst presence[10,11]. This inconsistency in decidualization exemplifies the translational benefits of studying cellular differentiation in human cell lines. In vitro assays through primary cell culture are often utilized to explore the modulation of decidualization via hormones[4,6,10]. However, limitations of this method can occur upon the obtainability of a large sample size of human tissue without significant variations in cycle phase and pregnancy history. Nevertheless, measures can be taken to ensure limitations are minimized by planning the study far enough in advance to guarantee ample samples and scheduling biopsies in the proliferative phase (Day 9 - Day 12) of the menstrual cycle.

In this study, HESC are cultured and artificially decidualized through a novel method first described in Brosens *et al.*[16] in which hormones E2, P4, and cAMP are supplemented to the culturing medium throughout a 6 day incubation period. Typically, alternative methods[12,14] primed cell cultures for artificial decidualization through the addition of E2 and MPA over an extended 14 day incubation period. The supplementation of cAMP to the culturing media synergistically amplifies the decidualization of ESC in a shortened time frame while simultaneously upregulating the expression of genes containing cAMP responsive element promoter regions (*i.e., PRL* and *IGFBP1*)[12,13]. This method, based upon the protocol described in Kommagani *et al.*[10], permits the optimization of the isolation and culture of HESC. Cell culture purity was optimized by utilizing a 40 µm cell strainer to separate stromal and epithelial cell lines. Furthermore, Ficoll-Paque PLUS reagent was applied to ensure a viable, high yield isolation of pure stromal cells upon the removal of blood cells from the sample. An additional centrifugation step (400 x g for 30 min) was included following Ficoll addition to ensure the complete elimination of blood cells from the cell population. Finally, HESC viability and yield were

optimized upon culturing cells until 95% confluency for decidualization. Altogether, these additional steps ensured the pure culture of viable, high yield HESC.

The degree of decidualization was assessed utilizing qRT-PCR, ELISA, and phalloidin staining to observe the cytoskeletal rearrangement and upregulation of hallmark genes. In qRT-PCR, the transcript levels of decidualization markers *PRL* and *IGFBP1* were assessed. Upon decidualization of HESC, *PRL* and *IGFBP1* levels increased in a time-dependent fashion, as established from previous research[6]. The time-dependent increase of secreted PRL levels was also confirmed by examining the HESC tissue culture media via ELISA. Furthermore, morphological alterations of stromal cells into decidual cells can be visualized through the costaining of actin filaments via phalloidin and nuclear marker 4',6-Diamidino-2-Phenylindole (DAPI)[17]. Together, these experimental results confirmed the effectiveness of culturing HESC through the supplementation of E2, MPA, and cAMP.

The capability of this protocol to study gene specific knockdown was also assessed utilizing siRNA against *SRC-2*. Morphologically, *SRC-2* transfected HESC remained fibroblastic, whereas HESC treated with control siRNA decidualized into epithelioid cells. *SRC-2* knockdown efficiency was assessed through the transcript level and was significantly decreased, indicating a successful silencing of specific gene with our protocol. Thus, our protocol can be a useful resource for investigators with an interest in delineating the role of specific gene(s) in endometrial decidualization.

While advancements in understanding the underlying molecular mechanisms of decidualization have been made, a significant knowledge gap still exists on the specifics. Improper decidualization has been established as a root cause for implantation failure and subsequent early embryo miscarriage[4,6,7,10]. Therefore, further exploration regarding the epigenetic factors and characterization of molecular pathways is necessary for the diagnosis and treatment of pregnancy failure.

In conclusion, the protocol presented throughout this study establishes an efficient method to isolate and culture a pure and viable line of human endometrial stromal cells. By supplementing hormones E2, MPA, and cAMP to the culturing media, artificial decidualization can be induced as confirmed by qRT-PCR, ELISA, and Phalloidin staining. Further, our protocol outlines detailed steps required for the efficient knockdown of a specific gene of interest using siRNA and lipid-based transfections. Altogether, this method presents the ability to study the underlying cellular and molecular mechanism(s) associated with HESC decidualization.

Disclosures

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