Effect of primary human endometrial stromal cells on epithelial cell receptivity and protein expression is dependent on menstrual cycle stage

A. Evron¹,², S. Goldman¹, and E. Shalev¹,²,*

¹Laboratory for Research in Reproductive Sciences, Department of Obstetrics and Gynecology, Ha’Emek Medical Center, Afula 18101, Israel
²Rappaport Faculty of Medicine, Technion, Haifa, Israel

*Correspondence address. E-mail: shaleve@technion.ac.il

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Introduction

Successful implantation requires a receptive endometrium, a normal and functional embryo at the blastocyst stage of development and a synchronized dialogue between maternal and embryonic tissues (Simon et al., 2000; Diedrich et al., 2007). Implantation occurs only in a limited time span of uterine receptivity, coined ‘window of implantation’ (Bourgain and Devroey, 2007; Diedrich et al., 2007). Ovarian steroids induce morphological and functional changes in the endometrium preparing it for successful implantation. These mediators of implantation embrace a large variety of inter-related molecules including adhesion molecules, cytokines, growth factors, lipids and others (Lessey, 2000; Lessey and Castelbaum, 2002; Lessey, 2004). Endometrial receptivity is ascribed to the epithelial cells and consists of the acquisition of adhesion ligands together with the loss of inhibitory components that may act as a barrier to an attaching embryo (Aplin, 2000). The importance of endometrial epithelial–stroma interactions in the acquisition of epithelial receptivity has been documented in previous studies (Bruner et al., 1995; Yang et al., 2001). Stromal regulation of the epithelium is especially evident in the cycling endometrium, which undergoes monthly developmental changes in response to ovarian steroids. Steroid receptors in the stromal cells are required for the action of estrogens and progesterone (Osteen et al., 1994; Bruner et al., 1995; Kurita et al., 1998; Young, 2007). Lessey et al. (2002) reported that progesterone induces the release of heparin-binding epidermal growth factor (HB-EGF) from endometrial stromal cells (ESC) which acts in a paracrine fashion on epithelial cells by increasing the expression of integrin beta3. Disturbances in the
Materials and Methods

Tissue collection

Endometrial tissue was obtained with informed consent from patients under the age of 35 years undergoing hysteroscopy as part of an infertility evaluation protocol. Included in this study were only those biopsies which were obtained from patients diagnosed as severe male factor infertility. Samples exhibiting endometrial pathologies were excluded. Endometrial biopsies were obtained in the menstrual cycle days 10–17 (before the window of implantation) and on cycle days 19–23 (during the window of implantation). Dating of the endometrial tissue was performed according to the date of last menstrual period, endocrine profile (LH, estradiol, progesterone) and standard histological dating (Noyes, et al., 1950).

For each experimental setting, primary ESC isolated from biopsies obtained from at least three different women were used in at least two independent experiments.

Patients

The study group was composed of women who had not been diagnosed with any known female causes of infertility, such as uterine fibroids, endometriosis, hydrosalpinx or other factors which might interfere with uterine receptivity, and in whom the infertility apparently results primarily or exclusively from a male factor. Women included in the study had not undergone more than three independent IVF cycles. All the women became pregnant and stayed pregnant until term or at least until the end of our study. Altogether, 14 women were enrolled (average age: 29.21 ± 0.87 years). In six women, biopsy was obtained prior to the window of implantation and in eight women during the window of implantation (Table I).

Isolation of human ESC

Tissues were cut into 0.5–1 mm pieces and incubated with 1 mg/ml of collagenase type IA (Sigma, St Louis, MO, USA) in M-199 medium (Kibbutz Beit-Ha’Emek, Israel) containing 10% fetal calf serum (FCS, Beit-Ha’Emek) and penicillin/streptomycin (Beit-Ha’Emek). Digestion was performed for 2 h at 37°C under gentle shaking (8g). The suspension was then transferred through a 25G syringe and cells were freed from collagenase by centrifugation for 10 min at 800g. The pellet was re-suspended in 1 ml M-199 and filtered through a 40 μm cell strainer into growth medium. We verified the purity of primary ESC by using immunohistochemistry with specific antibodies to vimentin (positive) and cytokeratin-7 (negative), Fig. 1A and B, respectively. Primary human ESC were seeded into plastic Petri dishes (BD Falcon, Franklin Lakes, NJ, USA) containing culture medium. We verified the purity of the isolated ESC by using immunohistochemistry with specific antibodies to vimentin (positive) and cytokeratin-7 (negative), Fig. 1A and B, respectively. Primary human ESC were cultured in M199 medium containing 10% FCS (Beit-Ha’Emek). We did not use charcoal-stripped FCS, because we thought it was unnecessary owing to the absence of steroid hormone production in

<p>| Table I Data (mean ± SEM) for the women who provided the endometrial biopsies from which primary ESC were obtained. |
|-------------------------------------------------|---------------------------------|-----------------|-----------------|---------------|</p>
<table>
<thead>
<tr>
<th>Number of women</th>
<th>Cause of infertility</th>
<th>Age (years)</th>
<th>Number of IVF cycles</th>
<th>Day of cycle</th>
<th>Histological dating</th>
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</thead>
<tbody>
<tr>
<td>Before window of implantation</td>
<td>6</td>
<td>Severe male factor infertility</td>
<td>28.5 ± 1.4</td>
<td>1.8 ± 0.3</td>
<td>15.8 ± 0.5</td>
</tr>
<tr>
<td>At window of implantation</td>
<td>8</td>
<td>Severe male factor infertility</td>
<td>29.7 ± 1.1</td>
<td>1.1 ± 0.3</td>
<td>20.1 ± 0.4</td>
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</table>
endometrial cell cultures. Stromal cell cultures were grown to confluence (2–3 days) in a humidified atmosphere containing 5% CO2 at 37°C and subsequently used (within Passage 1) for the creation of the endometrial 3D co-culture model, RNA extraction, immunofluorescence and zymography.

**Immunohistochemistry**

Immunohistochemistry was performed using the Histostain-Plus kit (Zymed Laboratories, Inc., USA). Isolated primary ESC grown on slides were fixed with cytospray (a common cell-fixation material replacing ethanol–acetone fixation) for 20 min and quenched with 3% hydrogen peroxidase in methanol to eliminate endogenous peroxidase activity. The slides were washed, blocked and incubated at room temperature with primary antibodies [mouse anti-human cytokeratin-7 (1:100, clone OVTL12/30, Biogenics) and mouse anti-human vimentin (1:200, clone V9, Zymed Laboratories). The secondary antibody used was Histostain-Plus broad-spectrum biotinylated secondary antibody (Zymed Laboratories). Negative controls were run routinely in parallel by replacing the primary antibody with mouse IgG antibody. No specific immunoreactivity was detected in the negative control specimens. Slides were then developed with a substrate–chromagen solution of aminoethyl carbazole (Zymed Laboratories).

**Cell cultures**

The human endometrial adenocarcinoma RL95-2 cell line (ATCC Catalog No. CRL-1671) is adhesive for JAR (human choriocarcinoma cell line) spheroids (Li et al., 2002, 2003) and considered a highly receptive endometrial cell-line model with a non-polarized cellular phenotype. RL95-2 cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) F:12 medium (Beit-Ha’Emek) containing 10% FCS and penicillin/streptomycin. The JAR (Jar, HTB 144) cell line was established from a trophoblast tumor of the placenta (1988 American Type Culture Collection Catalogue). JAR cells were cultured in M-199 medium containing 10% FCS and penicillin/streptomycin. Cell cultures were maintained in a humidified atmosphere containing 5% CO2 at 37°C. After reaching confluence, RL95-2 cells and JAR cells were harvested from plastic Petri dishes (Falcon) using trypsin/EDTA (Beit-Ha’Emek), centrifuged and re-cultured. Primary ESC were grown to confluence (cultured for 2–3 days) immediately after isolation. Cells were then released from the culture flasks by trypsin/EDTA and re-seeded for the experiments. All of the experiments were performed 48–72 h after re-seeding (within Passage 1).

**Creation of an endometrial 3D co-culture model**

For the creation of the endometrial 3D co-culture model, primary ESC (Fig. 1Ca) and RL95-2 cells (Fig. 1Cb), which were grown to confluence (2–3 days), were harvested from plastic Petri dishes (Falcon) by trypsin/EDTA and centrifuged for 10 min at 800g in order to remove trypsin. The pellet was re-suspended in 1 ml culture medium. Primary ESC were seeded into 24-well plates (Falcon) (for attachment and growth assay) or into 35 mm plastic Petri dishes (Falcon) (for RNA extraction) containing M199 culture medium at a concentration of 2 × 105 cells/ml. After incubation for 45 min in humidified atmosphere containing 5% CO2 at 37°C, culture medium was replaced with DMEM F:12 medium and RL95-2 cells were seeded onto the attached primary ESC at a concentration of 2 × 105 cells/ml. The endometrial 3D co-cultures were incubated in a humidified atmosphere containing 5% CO2 at 37°C. RL95-2 monolayer cultures, primary stromal cell monolayer cultures and endometrial co-cultures were cultured in medium containing 1.5% FCS in the presence or absence of progesterone (Sigma) for 48–72 h (Fig. 1D).

**XTT assay of cell number**

Culture media from endometrial cells were collected in order to evaluate cell proliferation. Cell count was performed with XTT Reagent kit (XTT, cell proliferation kit, Beit-Ha’Emek) according to the manufacturer’s protocol. This assay is based on the activity of mitochondrial enzymes in live cells, reducing tetrazolium salts, XTT, into colored formazan compounds which can be detected colorimetrically with a spectrophotometer at 450 nm (ELISA reader ELx808 Ultra Microplate Reader Bio-Tek).
Stroma cells affect endometrial epithelial receptivity

Instruments, Inc., Winooski, VT, USA). XTT absorbance is proportional to the number of cells in the culture.

Substrate-gel electrophoresis (zymography)

For the assessment of MMP secretion and activity in the conditioned medium of endometrial cell cultures, primary ESCbw or ESCw were cultured immediately after isolation in medium containing 10% FCS. The cultures were subsequently grown to confluence (2–3 days); primary stromal cells were released from the plastic Petri dishes by trypsin/EDTA and seeded into new flasks containing medium supplemented with 1.5% FCS in the presence and absence of progesterone. For the creation of the 3D co-culture model, primary stromal cells were incubated for 40 min, and then RL95-2 cells were seeded on top of the attached stromal cells. RL 95-2 monolayer culture, primary ESC (before the window of implantation or during the window of implantation) and endometrial 3D co-cultures were cultured in the presence and absence progesterone (0.1, 1, 10 μM) for 48 h.

Following this culture period, conditioned media (cm) were collected and subjected to substrate-gel electrophoresis (Zymography) on gels containing gelatin as the substrate in order to detect proteolytic enzyme activity in the CM. The CM was diluted in sample buffer (5% sodium dodecyl sulfate, 20% glycerol in 0.4 mol/l Tris, pH 6.8, containing 0.02% Bromophenol Blue without 2-mercaptoethanol) and electrophoresed, through a 10% polyacrylamide gel containing 0.5% gelatin (50 mg/ml). Afterwards, gels were washed twice in 2.5% Triton X-100 (Sigma) for 15 min and incubated for 24 h at 37°C in 0.2 mol/l NaCl, 5 mmol/l CaCl2, 0.2% Brij 35 and 50 mmol/l Tris, pH 7.5. The buffer was decanted and the gels stained with Coomasie Blue in 30% methanol and 10% acetic acid for 10 min at room temperature on Comfort shaker (Comfort Heto Master Shake, Heto-Holten A/S Type: SBD50-1, Paris, France) at 12g. Stain was washed out with water until clear bands were seen. Areas where proteolytic enzyme activity degraded the gelatin were seen as absence of staining. Identification of each gelatinase band was carried out in accordance with their molecular weight and commercial standards (gelatinase A and B, 7 μl; Oncogene Science, Cambridge, MA, USA, data not shown). These bands (proMMP) were quantified with the densitometer system, using Bio-Capt and TINA software (Raytest, Staubenhardt, Germany). MMP secretion was expressed as a percentage of control.

Attachment and invasion assays

Attachment of JAR spheroids to endometrial 3D co-culture and RL95-2 monolayer

For the JAR spheroid attachment and growth assay, primary ESCbw or ESCw were cultured immediately after isolation in medium containing 10% FCS. The cultures were subsequently grown to confluence (2–3 days) and cells were released from the plastic Petri dishes by trypsin/EDTA and seeded into new dishes containing medium supplemented with 1.5% FCS in the presence or absence of progesterone. For the creation of the 3D co-culture model, primary stromal cells were incubated for 40 min, and then RL95-2 cells were seeded on top of the attached stromal cells. Endometrial 3D co-cultures and RL95-2 monolayer culture were grown in DMEM F:12 culture medium containing 1.5% FCS in the presence and absence of progesterone (1 μM) for 48–72 h.

In order to investigate the possibility of a direct effect on JAR spheroids, progesterone was added to the spheroids alone as part of the control of the study.

For the attachment assays, JAR trophoblast spheroids were prepared and tested as described (John et al., 1993) with modifications: briefly, 1 x 10⁶ JAR cells per 10 ml M-199 medium, containing 10% FCS and penicillin/streptomycin, were agitated at 37°C on a Comfort shaker at 12g for 48 h. Prior to the addition of JAR spheroids onto endometrial cell cultures, culture medium of the endometrial 3D co-cultures and RL95-2 monolayer culture was replaced with M199 culture medium containing 1.5% FCS without progesterone. Thereafter, spheroids with a diameter of 100 ± 20 μm were gently delivered with a micro-denuding pipette (150 μm diameter) onto the confluent endometrial 3D co-culture, and in addition, onto the confluent monolayer of RL95-2 cells grown in 24-well culture plates. After 60 min incubation at 37°C, the culture plate was shaken aggressively at 15g (VORTEX GENIE, Scientific Industries, Chicago, IL, USA) for 60 min. The medium containing unattached spheroids was collected, and fresh medium was added to the wells. Spheroids remaining in each well were counted using a phase-contrast microscope. Spheroid attachment is expressed as a percentage of seeded spheroids. It is accepted that in attachment assays, n represents accumulated number of spheroids (Tinel et al., 2000; Heneweier et al., 2002). Each test represents at least four independent experiments, each in triplicate.

Growth of JAR spheroids on endometrial 3D co-culture and RL95-2 cell monolayer

Spheroid outgrowth was measured under the microscope for the next 96–168 h. Each spheroid diameter was measured using a special scale in the ocular (Fig. 1E).

Immunofluorescence staining

For immunofluorescence analysis, RL95-2 monolayer cultures, primary ESCbw/ESCw and endometrial 3D co-cultures were cultured in the absence and presence of progesterone (1 μM) on glass coverslips in 35 μl serum-free medium drops under mineral oil. Endometrial cell cultures were cultured under these conditions for 48–72 h. Cells were washed three times with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde (Electron Microscope Sciences, Belgr) in PBS for 10 min at 4°C, then washed twice with PBS and permeabilized for 5 min at 4°C with 0.1% Triton (Sigma) in PBS. After a PBS wash, slides were incubated for 1 h with blocking buffer [PBS supplemented with 3% bovine serum albumin (BSA)], then washed three times with PBS and incubated for 30 min at room temperature with primary antibodies (mouse anti-human PB-1 and mouse anti-human PR-B: Santa Cruz Biotech, CA, USA) 1 μg per slide in 700 μl PBS supplemented with 1.5% BSA. After five washings with PBS, slides were incubated for 30 min in the dark with secondary fluorescein-labeled antibody [for F-actin: phallolidin, AlexaFluor-488, A-12379; for PB-1 and PR-B: goat-anti-mouse immunoglobulin (IgG) conjugated with AlexaFluor-633; all from Molecular Probes], 0.5 μg per slide in 700 μl PBS supplemented with 1.5% BSA. Negative controls were run in parallel by replacing the primary antibody with mouse IgG isotype control antibody (Santa Cruz Biotech) at the same concentration as the primary antibody (1 μg per slide in 700 μl PBS supplemented with 1.5% BSA). No specific immunoreactivity was detected in these negative control specimens. Following three washings with PBS, stained cells were photographed using a confocal microscope (the confocal system is composed of a BioRad radiance 2000 confocal set-up hooked to an upright fluorescence microscope Nikon E600 with a ×60 lens). The photos were analyzed by Image Pro software (Media Cybernetics, Bethesda, MD, USA), which quantifies density per area.

RNA extraction

RL95-2 monolayer culture, primary ESC (before the window of implantation/during the window of implantation) and endometrial 3D co-cultures were cultured in the absence and presence of progesterone (1 μM) for 48–72 h. Total cellular RNA from cell cultures was extracted using a total RNA isolation kit EZ-RNA (Beit-Ha’Emek) according to
manufacturer's instructions. RNA concentration was determined spectrophotometrically.

**First-strand cDNA synthesis for RT–PCR**

In order to obtain the cDNA, RNA (5 μg) was denatured at 70°C for 10 min and then reverse-transcribed in the presence of 25 ng/μl random primer (Promega, Mannheim, Germany), 2.5 mM MgCl₂, 0.5 mM deoxy-NTPs, 10 nM dithiothreitol and 10 U ribonuclease H-reverse transcriptase (SuperScript II RT, Life Technologies, Inc., Invitrogen, Dorset, UK) for 60 min at 42°C, and 5 min at 95°C. Subsequently, 10 μl of the resulting cDNA was used as a template for PCR.

**RT–PCR**

The first-strand cDNA product, 10 μl, was subsequently amplified in a total volume of 50 μl, containing 1.5 U Taq DNA polymerase (Sigma), 200 μM dNTPs (Promega), 2 mM MgCl₂, 50 pmol of each primer and buffer (10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100). Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PB-1 and PR-B were used. The PCR amplification for all sets of primers was performed for 30 cycles in an automated thermocycler profile. Before starting, each primer was normalized for sub-saturation conditions. We used a series of dilutions of a bulk RNA preparation to determine sub-saturation conditions for the PCR products using GAPDH. The amplification parameters were denaturation at 94°C for 5 min, followed by 30 cycles as follows: denaturation at 94°C for 30 s, annealing at 58°C for 1 min and elongation at 72°C for 90 s and a 72°C extension for 10 min. RT–PCR products were analyzed by 2.5% agarose ethidium bromide gel electrophoresis. Images were captured on Polaroid film (Hertfordshire, UK) under UV light. Products were quantified using a densitometer system with TINA software (Raytest). Amplification of the housekeeping gene GAPDH transcripts was performed simultaneously to confirm RNA integrity and PCR efficiency and for the quantification of cDNA. Negative control reactions containing samples without cDNA or Taq enzyme were used.

**Primer design**

The sequences of appropriate primers were obtained from the gene bank and prepared at IDT, Inc., Hy-Labs, Rehovot, Israel. GAPDH (GenBank accession no. M33197)

- **GAPDH FWD 5′-TGATGACATCAAGAAGGTGGTGAAG-3′**
- **GAPDH REV 5′-TGCGAGGGCCCATGCACCT-3′**
- 230 bp product.

PB1 (GenBank accession no. F09621)

- **PB1 FWD 5′-GCAGTGTGGTTATCCTTTAATGCAA-3′**
- **PB1 REV 5′-CCACTCAAAAACTGAACCCCT-3′**
- 150 bp product.

PR-B (GenBank accession no. M15716)

- **PRB FWD 5′-ACACCTTGCTGGAAGTTTCG-3′**
- **PRB REV 5′-TGAGCTCTTTTCAGGGGCAT-3′**
- 196 bp product.

**Statistical analysis**

Results are expressed as mean ± SEM. Student’s t-test was used for the statistical comparisons of spheroid growth, PB-1 and PR-B protein expression and PB-1 and PR-B gene expression. χ² test was applied for analysis of the attachment assay, and one-way analysis of variance was used for analysis of the zymopraphy results. P < 0.05 was considered statistically significant.

**Results**

**Basal proMMP secretion profile in endometrial cell cultures not treated with progesterone**

proMMP-2 and proMMP-9 secretion was detected in all endometrial cell cultures not treated with progesterone (control). proMMP-2 was the most abundant gelatinase secreted by these endometrial cell cultures (684.2 ± 51.72 for proMMP-2 versus 263.5 ± 25.1 for proMMP-9 in RL95-2 cells, P = 0.0005; 1764 ± 95.4 for proMMP-2 versus 280.33 ± 47.6 for proMMP-9 in primary ESCbw, P = 0.0001; 2285.33 ± 171.6 for proMMP-2 versus 57.67 ± 1.5 for proMMP-9 in RL/ESCbw co-culture, P = 0.0004; 6607.25 ± 462.4 for proMMP-2 versus 1422.67 ± 175.1 for proMMP-9 in primary ESCw, P = 0.0004 and 3933 ± 135.5 for proMMP-2 versus 1897.67 ± 336.3 for proMMP-9 in RL/ESCw co-culture, P = 0.008, Fig. 2). proMMP-2 and proMMP-9 secretion was significantly higher in primary ESCw and RL/ESCw co-culture compared with all other endometrial cell cultures, P < 0.05, Fig. 2.

**Dose-dependent effect of progesterone on proMMP-2 and proMMP-9 secretion in endometrial cell cultures**

We found that untreated endometrial cell cultures secreted proMMPs. We determined this secretion to be 100% for the purpose of comparing cell responsiveness to progesterone. Deviations in the percentage from 100% are the result of the effect of progesterone. Results of three independent experiments are depicted in Fig. 3.

In primary ESCbw, 0.1, 1 and 10 μM progesterone significantly increased proMMP-2 secretion (114.99 ± 2.13% for 0.1 μM, P = 0.038; 112.49 ± 3.73% for 1 μM, P = 0.044 and 124.56 ± 3.71% for 10 μM, P = 0.0046, all versus control, Fig. 3); in primary ESCw, 0.1 and 10 μM progesterone significantly decreased proMMP-9 secretion (66.78 ± 1.185%, 0.1 μM progesterone, P < 0.0005; 57.5 ± 1.52%, 10 μM progesterone, P = 0.0001, all versus control, Fig. 3). Progesterone, at all concentrations tested, had no significant influence on proMMP-2 and proMMP-9 secretion in RL95-2 cell culture, RL/ESCbw co-culture and RL/ESCw co-culture (Fig. 3). No proMMP-9 secretion was detected in RL/ESCw co-culture and primary ESCbw (results not shown).

**The effect of primary ESC on JAR spheroid attachment and growth in endometrial 3D co-culture**

JAR spheroid attachment rates to RL95-2 were high and progesterone had no influence on attachment rates. The presence of primary ESCbw (cycle days 10–17) significantly reduced JAR spheroid attachment to overlying RL95-2 cells (45.11 versus 75.82%, P < 0.0001, χ² = 51.154). Progesterone treatment to the RL/ESCbw co-culture led to a significant increase in JAR spheroid attachment rate (70.63 versus 45.11% P = 0.00004, χ² = 20.363). The presence of primary ESCw (cycle days 19–24) had no influence on JAR spheroid attachment to overlying RL95-2 cells, and progesterone treatment did not influence attachment rates in this endometrial co-culture. In order to study a possible direct effect of progesterone on spheroids,
spheroid attachment rate to plastic was measured. JAR spheroid attachment rates to plastic were low, and progesterone had no influence on attachment rates (Table II and Fig. 4).

Spheroid diameter increased in spheroids grown on endometrial cells and on plastic during the whole culture period, with the most significant differences in spheroid diameter between control and progesterone treatment groups observed between 24 and 96 h (Fig. 5). The diameters of spheroids grown on plastic were significantly smaller in progesterone-treated spheroids compared with untreated spheroids after 96 h (mean diameter: 151.54 ± 11.7 versus

**Figure 2** Basal pro-MMP secretion (control). Mean ± SEM optical density of proteolytic bands in gel, n = 3 experiments. Black, proMMP-2; white, proMMP-9. RL, RL95-2 monolayer culture; Str.b.w, primary ESC obtained before the window of implantation; RL/Str.b.w, RL95-2 cells cultured on top of Str.b.w; Str.W, primary ESC obtained during the window of implantation; RL/Str.W, RL95-2 cells cultured on top of Str.W. *P < 0.05 for proMMP-2 versus proMMP-9; **P < 0.05 for Str.W and RL/Str.W versus RL, Str.b.w. and RL/Str.b.w.

**Figure 3** Dose–response effect of 48 h to progesterone (0.1, 1, 10 µM) treatment on proMMP-2 and proMMP-9 secretion from n = 3 experiments. (A) Representative proteolytic bands of proMMP-2 and proMMP-9 in gel. (B) Mean ± SEM of percentage optical density (of proteolytic bands in gel); white, control (without any treatment); dashed, 0.1 µM progesterone; black, 1 µM progesterone; dotted, 10 µM progesterone; *P < 0.05 (progesterone treated versus untreated).
In spheroids grown on RL95-2 monolayer culture, a significant difference in spheroid diameter between the control group (mean diameter: 160.52 ± 8 mm, P = 0.005, Fig. 5A) and the progesterone-treated group (mean diameter: 207.33 ± 8.2 mm, P = 0.0004, Fig. 5B) was observed after 96 h. Diameters of spheroids grown on progesterone-treated endometrial co-cultures with primary stromal cells before and during the window of implantation were significantly larger, already after 24 h, compared with untreated endometrial co-cultures (mean diameter: 143 ± 4.4 versus 128.67 ± 3.6 μm, P = 0.001, Fig. 5C; 102 ± 4.7 versus 77.4 ± 4.6 μm, P = 0.0009, respectively, Fig. 5D).

### Table II JAR spheroid attachment to endometrial cells and plastic.

<table>
<thead>
<tr>
<th></th>
<th>Not treated: number of total seeded spheroids</th>
<th>Not treated: number of attached spheroids (%)</th>
<th>Progesterone: number of total seeded spheroids</th>
<th>Progesterone: number of attached spheroids (%)</th>
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<tr>
<td>Spheroids on plastic</td>
<td>65</td>
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<td>19 (27.9)</td>
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<td>Spheroids on RL95-2</td>
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<td>116 (75.8)</td>
<td>149</td>
<td>122 (81.8)</td>
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<td>Spheroids on RL/Str.b.w (number of different women = 3)</td>
<td>133</td>
<td>60* (45.1)</td>
<td>143</td>
<td>101** (70.6)</td>
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<tr>
<td>Spheroids on RL/Str.w (number of different women = 3)</td>
<td>172</td>
<td>140 (81.3)</td>
<td>199</td>
<td>172 (86.43)</td>
</tr>
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</table>

RL95-2, RL95-2 monolayer culture; RL/Str.b.w, RL95-2 cells cultured on top of primary stromal cells obtained before the window of implantation; RL/Str.w, RL95-2 cells cultured on top of primary stromal cells obtained during the window of implantation. Numbers in parentheses depict percentage of attached spheroids.

*P < 0.0001; χ² = 51.154.

**P < 0.00004; χ² = 20.363.

### Expression of PB-1 protein in primary ESC, RL95-2 monolayer culture and endometrial 3D co-culture

A significantly lower PB-1 protein expression, as observed using antibody detection, was observed in untreated RL/ESCbw in comparison with untreated RL95-2 (RL) culture (IOD per area: 0.103 ± 0.002 versus 0.149 ± 0.022; P = 0.038, Fig. 6). Progesterone treatment of the RL/ESCbw co-culture led to a significant increase in PB-1 expression (IOD per area: 0.178 ± 0.02 versus 0.103 ± 0.002; P = 0.0385, Fig. 6). Progesterone significantly increased PB-1 protein expression also in primary ESCbw (IOD per area: 0.38 ± 0.22 versus 0.15 ± 0.041; P = 0.049, Fig. 6).

### PB-1 gene expression in primary ESC culture, RL95-2 monolayer culture and endometrial 3D co-culture

Progesterone significantly increased PB-1 mRNA levels in RL95-2 monolayer culture (0.99 ± 0.1 versus 0.62 ± 0.09, PB-1/GAPDH ratio ± SEM, P = 0.04), Fig. 7. The presence of primary ESCbw had no significant influence on PB-1 mRNA levels in RL/St.b.w co-culture. Progesterone significantly increased PB-1 mRNA levels in RL/St.b.w (0.81 ± 0.037 versus 0.46 ± 0.017, P = 0.006). PB-1 mRNA levels...
in untreated primary ESCw were significantly higher compared with untreated primary ESCbw, *P = 0.01, Fig. 7. Progesterone had no influence on PB-1 mRNA levels in primary ESCw and RL/ESCw co-culture.

**PR-B isoform protein in endometrial cells**

Progesterone significantly increased PR-B protein expression in RL95-2 monolayer culture (integrated optical densities, IODs, per area: 3.788 ± 1.617 for PR-B in progesterone-treated RL95-2 monolayer culture versus IOD per area: 0.141 ± 0.0197 for PR-B in untreated RL95-2 monolayer culture, *P = 0.043, Fig. 8). Progesterone had no significant influence on PR-B in all other cultures. In untreated RL/ESCbw co-culture, PR-B distribution was significantly higher compared with untreated RL95-2 monolayer culture (IOD per area: 2.709 ± 0.82 in untreated RL/ESCbw co-culture versus IOD per area: 0.141 ± 0.0197 in untreated RL95-2 monolayer culture, *P = 0.023, Fig. 8). PR-B distribution was also significantly higher in untreated primary ESCw compared with untreated RL/ESCbw co-culture (IOD per area: 4.68 ± 0.385 in untreated primary ESCw versus IOD per area: 0.182 ± 0.033 in RL/ESCw co-culture, *P = 0.039, Fig. 8).

**PR-B mRNA level in primary ESC culture, RL95-2 monolayer culture and endometrial 3D co-culture**

Progesterone significantly increased PR-B mRNA levels in RL95-2 monolayer culture (PR-B/GAPDH ratio: 2.795 ± 0.49 in five independent experiments for progesterone-treated RL95-2 monolayer culture versus PR-B/GAPDH ratio: 0.479 ± 0.08 in four independent experiments for untreated RL95-2 monolayer culture, *P = 0.007, Fig. 9). In untreated RL/ESCbw co-culture, PR-B mRNA levels were significantly higher compared with untreated primary ESCbw (PR-B/GAPDH ratio: 0.758 ± 0.06 in five independent experiments for untreated RL/ESCbw co-culture versus PR-B/GAPDH ratio: 0.0486 ± 0.02 in three independent experiments for untreated primary ESCbw, *P = 0.02, Fig. 9). In untreated primary ESCw, PR-B mRNA levels were significantly higher compared with untreated primary ESCbw (PR-B/GAPDH ratio: 0.891 ± 0.04 in three independent experiments for untreated primary ESCw versus PR-B/GAPDH ratio: 0.486 ± 0.02 in three independent experiments for untreated primary ESCbw, *P = 0.001, Fig. 9). Progesterone significantly decreased PR-B mRNA levels in primary ESCw (PR-B/GAPDH ratio: 0.318 ± 0.05 in three independent experiments for progesterone-treated primary ESCw versus PR-B/GAPDH ratio: 0.891 ± 0.04 in three independent experiments for untreated primary ESCw, *P = 0.0009.

*Figure 5* Changes in mean ± SEM spheroid diameter in progesterone (1 μM)-pre-treated endometrial cell cultures and progesterone (1 μM)-treated spheroids on plastic (pink line) versus untreated control (blue line). Spheroids were grown on (A) plastic, *P = 0.005; (B) RL95-2 monolayer culture, *P = 0.0004; (C) RL95-2/Stroma before the window of implantation (RL/St.b.w) co-culture, *P = 0.01; (D) RL/Stroma w co-culture, *P = 0.0009.
Figure 6  (A) Representative immunofluorescence pictures of plexin-B1 (PB-1) (blue) and F-actin (green) distribution in ESC following 1 μM progesterone (prog.) treatment for 48–72 h versus control (cont.). F-actin staining was used in order to depict the general cell shape, organization of the F-actin cytoskeleton and localization of PB-1 expression in the corresponding cells.  (B) Mean ± SEM IOD per area of at least n = 3 experiments. Black, control (with no treatment); dashed, progesterone treatment; **P < 0.05 for untreated RL95-2 versus untreated RL/Str.b.w.

Figure 7  Plexin B1 (PB-1) mRNA levels in endometrial cell cultures.  (A) Representative bands of PB-1 and GAPDH in 2.5% agarose gel.  (B) Mean ± SEM PB-1 to GAPDH mRNA ratio from at least n = 3 experiments. White, untreated control; black, progesterone (1 μM) treatment for 48–72 h. Product size: PB-1, 150 bp; GAPDH, 230 bp. **Versus untreated Str.b.w, P = 0.01.
Discussion

The problematic translatability of results from animal models to human implantation has led to the development of in vitro models using...
human cell-culture systems. Several studies investigating human implantation were conducted with choriocarcinoma spheroids on an endometrial epithelial cell line in monolayer cultures (Tinel et al., 2000; Hohn and Denker, 2002; Thie and Denker, 2002; Heneweer et al., 2005; Harduf et al., 2007). These studies have brought insights about molecular mechanisms during the early phases of implantation. The use of cell lines is appropriate for the investigation of molecular mechanisms, as they are available almost without restriction, enable controlled experimental manipulations and have known cell identity. Owing to the loss of original qualities, molecular mechanisms studied in cell lines need further investigations in primary cell cultures, where results obtained may more closely reflect in vivo conditions compared with those obtained from cell lines. Simon et al. (1997) demonstrated human blastocyst interaction with human primary endometrial epithelial monolayer culture, but endometrial stroma–epithelial interactions were not considered in these studies. The use of monolayer cultures does not take into account the importance of cell interactions. Endometrial biopsies taken from different phases of the menstrual cycle revealed important insights into the cyclic expression of the MMP system (Rodgers et al., 1994; Curry and Osteen, 2003; Goffin et al., 2003), PR isoforms (Lessey et al., 1988, 1996; Mote et al., 1999), receptivity markers (Hey et al., 1994; Acosta et al., 2000; Lessey et al., 2000; Lessey, 2003) and gene profiles during the implantation window (Kao et al., 2002; Riesiewijk et al., 2003). Biopsies tend to reflect in vivo conditions, but leave no ability for controlled experimental manipulations. In endometrial explant cultures, tissue integrity is sustained, and controlled experimental manipulations are possible, but the contribution of a certain cell type cannot be identified because of the inability to separate the explant into single components (Vassilev et al., 2005; Ponnampalam and Rodgers, 2006; Gaide Chevronnay et al., 2008). More and more studies investigating endometrial function are performed in alternative endometrial 3D co-culture models (Arnold et al., 2001; Pierro et al., 2001; Yang et al., 2001, 2002; Goffin et al., 2002; Bläuer et al., 2005; Cheng et al., 2007).

We developed an alternative in vitro endometrial co-culture model which enables the investigation of the influence of primary ESC on endometrial epithelial cells. The exact number of days that are required to lose the influence of cycle stage in in vitro cultured primary stromal cells is not known. It is, however, accepted that primary cell cultures retain their original characteristics over the first three to four passages and thereafter start to change. In this study, immediately after isolation, primary stromal cells were grown to confluence (culture for 2–3 days), released from the culture flask by trypsin and then re-seeded for the experiments. All of the experiments were performed 48–72 h later. We chose the endometrial adenocarcinoma cell line RL95-2 as a constant parameter on which the influence of stromal cells was tested. The RL95-2 cells are adhesive for JAR spheroids and have a non-polarized phenotype (Thie et al., 1996; Li et al., 2002, 2003). A number of studies on trophoblast–epithelium interaction and epithelial receptivity were performed with RL95-2 cells (Thie et al., 1996, 1997, 1998; Tinel et al., 2000; Heneweer et al., 2002; Harduf et al., 2007). In our alternative in vitro co-culture model, RL95-2 cells were seeded on top of primary ESC which were attached to plastic. In this alternative in vitro co-culture model, primary ESC are in direct cell-to-cell contact with the overlying endometrial epithelial cell line RL95-2: this was observed in photographs of the co-culture taken using a light microscope and in immunofluorescence pictures of F-actin-stained co-cultures. The filamentous F-actin, composed of the monomeric G-actins, is found in all eukaryotic cells and undergoes dynamic rearrangement through the polymerization of G-actin and breakdown of the filamentous F-actin into its monomers. We used F-actin staining in order to depict the general cell shape and organization of the F-actin cytoskeleton in our cell cultures. This enabled us to distinguish between the different cell types and to detect phenotypical changes through the re-organization of the F-actin cytoskeleton as a result of progesterone stimulation or prolonged in vitro culture. We did not consider the contribution of extracellular matrix in our in vitro co-culture model. In order to test whether our alternative endometrial co-culture model is suitable for further research, we tested proMMP secretion in the presence and absence of progesterone. We decided to test the progesterone responsiveness of our endometrial co-culture model in a dose–response experiment. Progesterone concentrations were chosen according to studies performed in our laboratory (Harduf et al., 2009). Progesterone is essential for the acquisition of uterine receptivity, embryo implantation and the maintenance of pregnancy. Several studies reported the involvement of the MMP system in endometrial tissue turnover and their control by ovarian steroids (Rodgers et al., 1994; Curry and Osteen, 2001, 2003; Goffin et al., 2003; Osteen et al., 2003; Goldman and Shalev, 2004; Vassilev et al., 2005).

In all of the untreated endometrial cell cultures, proMMP-2 secretion was dominant. We found that stromal cells from different phases of the menstrual cycle respond differently to progesterone in their gelatinase secretion profile. Progesterone suppression of stromal MMP expression during the luteal phase of the menstrual cycle agrees with former studies (Osteen et al., 1994; Curry and Osteen, 2001; Goffin et al., 2003); however, in these previous studies, in contrast to our findings, progesterone caused gelatinase suppression in epithelial cells. The lack of response can be attributed to the fact that RL95-2 cells are endometrial adenocarcinoma cells, which lost their progesterone responsiveness to gelatinase secretion (Ren et al., 2007). The presence of RL95-2 cells in co-culture abolished the progesterone-induced influence on gelatinase secretion observed in primary ESC. These results agree with those of Zhang et al. (2000) in which MMP-2 mRNA was constantly expressed in human endometrium during the menstrual cycle. In our study, only proMMP secretion and not gene expression was investigated and we cannot conclude whether there is an influence on MMP gene expression. Our results are in line with those of Goffin et al. (2002), who found that epithelial cells increased stromal MMP-2 activity, but had no influence on stromal proMMP-2 secretion. Zymography is a technique which tests proMMP secretion, and in order to investigate MMP activation specifically, one has to use other techniques, such as avidin staining. This was, however, not in the frame of our current study.

Several studies investigating blastocyst attachment were performed with choriocarcinoma spheroids on epithelial cell-line monolayer cultures. In these models, the choriocarcinoma spheroids mimic the trophoblast. In such trophoblast–endometrial epithelium interaction models, important insights about molecular mechanisms during blastocyst attachment were obtained, such as the remodeling of the F-actin cytoskeleton and RhoA distribution in endometrial epithelial cells.
upon contact with the trophoblast (Heneweer et al., 2002, 2005), repulsive and adhesive forces measured during trophoblast attachment (Thie et al., 1998) and the involvement of endometrial calcium channels (Tinel et al., 2000) and PB-1 (Harduf et al., 2007) in the attachment process. In all these studies, however, the influence of endometrial epithelial–stroma interactions on trophoblast attachment was not considered.

The present study was designed to test the influence of primary ESC on epithelial receptivity and trophoblast–endometrium interactions. Our results suggest that the effect of primary ESC on epithelial cell receptivity depends on whether the ESC were taken before or during the window of implantation. The maintenance of high epithelial cell receptivity in co-cultures containing primary ESCw seems to result from their in vivo exposure to progesterone. In vivo progesterone exposure probably leads to the activation of certain signaling pathways in endometrial cells, which were preserved during in vitro culture of primary ESC. Therefore, it seems that primary ESCw are already activated by progesterone. This may explain why there is no observed progesterone responsiveness in co-cultures containing primary ESCw, i.e. because the maximal response to progesterone has already been achieved.

The importance of endometrial epithelial–stroma interactions in the acquisition of epithelial receptivity has been documented in previous studies (Bruner et al., 1995; Yang et al., 2001). Lessey et al. (2002) has demonstrated that progesterone, acting through endometrial stromal PRs, induces HB-EGF release from stromal cells. HB-EGF acts in a paracrine way on epithelial cells by increasing the expression of integrin beta3 and thus increases epithelial receptivity.

In the present study, progesterone treatment of the co-culture containing primary stromal cells obtained before the window of implantation restored attachment rates to overlying highly receptive RL95-2 cells. This is consistent with the clinical increase in embryo implantation rates observed in women undergoing IVF-embryo transfer in which i.m. progesterone treatment was started 1 day after oocyte retrieval (Propst et al., 2001), and in the randomized, prospective study performed by Williams et al. (2001), which compared ongoing pregnancy rates in IVF-embryo transfer cycles with different starting points of progesterone treatment. Regarding spheroid growth rates, we observed rapid growth of JAR spheroids grown on endometrial co-cultures containing primary stromal cells obtained during the window of implantation, compared with co-cultures containing primary stromal cells obtained before the window of implantation and RL95-2 monolayer culture. Progesterone exerted no spheroid growth-promoting effect in the co-culture containing primary stromal cells obtained during the window of implantation, but showed a growth-promoting effect on spheroids cultured on co-cultures containing primary stromal cells obtained before the window of implantation and RL95-2 monolayer culture. Spheroids grown on epithelial cells have to dislodge cells in order to spread. We speculate that in primary stromal cells obtained during the window of implantation, certain signaling pathways are already activated (following exposure to ovarian steroids in vivo), influencing epithelial cells in their growth-promoting effect on trophoblast outgrowth. On the other hand, the presence of primary stromal cells obtained before the window of implantation in the co-culture decreases spheroid growth rate moderately with spheroids cultured on RL95-2 monolayer culture. This may result from spheroid factors exerting spheroid growth-inhibiting effects on overlying epithelial cells during the time before the window of implantation, thus preventing embryo implantation at an inappropriate time under physiological conditions. We observed that spheroids cultured on progesterone-pre-treated co-cultures containing primary stromal cells obtained before the window of implantation showed a steady growth which was higher at 24 and 96 h of spheroid culture compared with spheroids grown on untreated co-culture. Progesterone-treated primary stromal cells obtained before the window of implantation may acquire a more ‘during window of implantation’-like phenotype and thus exert spheroid growth-promoting effects on overlying epithelial cells. Another mechanism may involve a direct effect of progesterone on epithelial cells. It is important to note that all endometrial cells were pre-treated with progesterone for 42–72 h, after which medium was removed and replaced with trophoblast growth medium without progesterone. JAR spheroids were then gently delivered onto the endometrial cell cultures. Thus, the effect of progesterone may be retained in endometrial cell cultures for only 96 h before being lost and this may explain the lack of progesterone effect on spheroid growth at later time points (results not shown).

We are aware that our alternative in vitro endometrial 3D co-culture model and experimental setting do not reflect exact in vivo conditions. First, we used an epithelial cell line and not primary endometrial epithelial cells. This gave us the advantage of testing stromal influence on a constant epithelial parameter, but on the other hand ‘moved us away’ from the in vivo condition. Second, in our experimental set up, only endometrial cells and not JAR spheroids were exposed to progesterone. In vivo, trophoblast is exposed to ovarian steroids and it is reported that progesterone concentrations at the fetomaternal interface are higher compared with maternal serum progesterone concentrations (Li et al., 2003). Third, we used JAR choriocarcinoma spheroids in order to mimic trophoblast. JAR human choriocarcinoma cell line was established from a trophoblast tumor of the placenta (1988 American Type Culture Collection Catalogue) and therefore does not behave exactly like in vivo trophoblast. In JAR, spheroid inner cell mass (ICM) is not present and therefore in our study the contribution of ICM to trophoblast–endometrium interactions was not considered. A further limitation of our endometrial in vitro model is that stromal factors might exert a positive effect on trophoblast attachment. This is, however, difficult to detect in our model system owing to the fact that RL95-2 cells are inherently very adhesive for JAR spheroids. In order to test a possible positive effect of stromal factors exerted on trophoblast attachment, one can create an endometrial in vitro co-culture model using an endometrial cell line which is known to be of low adhesive potential to JAR spheroids, such as HEC-1A (Harduf et al., 2007).

In our study, the presence of primary stromal cells obtained before the window of implantation in endometrial co-cultures significantly decreased PB-1 protein expression compared with untreated RL95-2 monolayer culture. Immunofluorescence pictures revealed that this decrease in PB-1 protein expression occurred both in RL95-2 cells and primary stromal cells. Progesterone treatment significantly increased PB-1 mRNA and protein levels, observations that are consistent with our results obtained in the JAR spheroid attachment assays. Thus, a possible role for PB-1 in the trophoblast-epithelial endometrial adhesion process is suggested. Epithelial cells have an apical plasma membrane that is normally repellent and does not...
allow opposing uterine or embryonic cells, such as trophoblast, to adhere. At the time of implantation, however, uterine epithelial cells are reprogrammed toward adhesiveness for trophoblast (Thie et al., 1996; Aplin, 2000). It has been proposed that this process may be under the control of master genes, which regulate expression of the polarized epithelial phenotype and can prepare the apical cell pole of uterine cell contact with trophoblast (Horcajadas et al., 2004). It was suggested that there is a correlation between PB-1 expression and estrogen responsiveness in breast cancer cells (Rody et al., 2007), suggesting a possible involvement of steroid hormones in PB-1 regulation. Harduf et al. (2007) demonstrated that PB-1 is involved in JAR spheroid attachment to endometrial epithelial cells. The difference between gene and protein expression could be explained either by the sensitivity of each technique used or by post-transcription mechanisms. In a more recent study from our laboratory, PB-1 expression was documented in human endometrial tissue with a significant increase in expression found at the period corresponding with the implantation window (Amir et al., 2009). These findings suggest a role for the PB-1 receptor in the attachment stage of human embryonic implantation.

In our study, we investigated the protein distribution and mRNA levels for PR-B in endometrial cells. We demonstrated that PR-B protein distribution and gene expression are influenced by the interplay between RL95-2 epithelial cells and stromal cells obtained during different phases of the menstrual cycle. In untreated RL/ESCw co-cultures, we observed a significant decrease in PR-B protein expression compared with primary ESCw. Such a decrease was not observed at the mRNA level: this difference between protein and mRNA may be a result of differences in the sensitivity of each technique applied or post-transcription mechanisms. In studies performed by other groups, PR was identified in the nuclei of epithelial cells, stromal cells and myometrial smooth muscle cells of the uterus. The PR content of endometrial epithelium and stroma varies with the menstrual cycle. The epithelium demonstrated strong PR expression during the proliferative phase and in the early secretory phase, whereas at the post-ovulation phase it decreased sharply (Mote et al., 1999; Petersen et al., 2005; Kurihara et al., 2007). It was not possible in the current study to investigate the ratio between PR-A and PR-B expression in our model system but it is well documented that the relative abundance of PR subtypes may have important clinical consequences. Both the A and B isoforms of PR are capable of binding progesterone, forming a dimer and interacting with progesterone-responsive elements.

A growing body of evidence has accumulated in recent years, demonstrating that the PR-A and PR-B proteins are functionally different, regulating different physiological target genes in response to progesterone, and each protein may display different transactivation capabilities in different target tissues (Conneely and Lydon, 2000; Richer et al., 2002).

Conclusions

Primary ESC have an effect on epithelial cell receptivity and trophoblast–endometrial interactions that depends on menstrual cycle stage. A positive effect on attachment was found when ESC were obtained during the window of implantation. Nevertheless, only ESC obtained before the window of implantation were responsive to exogenous progesterone. This may be of clinical importance in the timing of progesterone application during IVF-embryo transfer. Endometrial epithelial–stroma interactions influence PB1 expression. Low levels of PB-1 may be responsible for low trophoblast attachment to endometrial epithelial cells.

Authors’ roles

A.E. performed the laboratory work, participated in the analysis and wrote the manuscript as part of her MSc thesis in the Technion under the supervision of E.S.; S.G. participated in conceiving and the design, supervised the laboratory work, analyzed the results and revised the manuscript critically. E.S. conceived and designs the study, analyzed the results and edited critically the paper. All three authors gave final approval.

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Stroma cells affect endometrial epithelial receptivity


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