A novel model of human implantation: 3D endometrium-like culture system to study attachment of human trophoblast (Jar) cell spheroids

Hai Wang1,†, Federica Pilla2,†, Sandra Anderson1, Sebastián Martínez-Escribano3, Isabel Herrer3, Juan M. Moreno-Moya3, Sirisha Musti2, Silvina Bocca1, Sergio Oehninger1,†, and José A. Horcajadas3,4,*, †

1The Jones Institute for Reproductive Medicine, Norfolk, VA 23507, USA 2Graduate Program in Public Health, Eastern Virginia Medical School, Norfolk, VA 23507, USA 3Fundación IVI (FIVI), Instituto Universitario IVI (IUIVI), University of Valencia, Valencia, Spain 4Araid at I+CS, Hospital Miguel Servet, Zaragoza, Spain

*Correspondence address. E-mail: jahorcajadas.iacs@aragon.es
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ABSTRACT: There is an urgent need to develop optimized experimental models to examine human implantation. These studies aimed to (i) establish a human endometrium-like three-dimensional (3D) culture system, and (ii) examine the attachment of trophoblast-like Jar spheroids to the culture. In the present work, 3D endometrial cultures were constructed with fibrin-agarose as matrix scaffold, and using epithelial and stromal cells from both human primary cultures and established cell lines. An attachment assay between trophoblast cells and the 3D culture was developed. Epithelial cells (cytokeratin+) concentrated on top of the matrix forming a monolayer, and stromal cells (vimentin+) resided within the matrix, resembling the normal endometrial structure. The capability of primary epithelial cells to form glands spontaneously was observed. Human trophoblast cells (Jar cells) were hCG+ by immunostaining, allowed to form spheroids, and confirmed to secrete hCG into the medium. Time-dependent experiments demonstrated a high rate of attachment of Jar spheroids to the epithelium, and adhesion was strongly related to the various cell types present in the 3D culture. An architecturally and functionally competent 3D endometrial culture system was established, that coupled with Jar spheroids mimicking trophoblast cells, provides a unique in vitro model for the study of certain aspects of human implantation.

Key words: attachment / 3-dimensional culture system / Jar spheroid / human endometrium

Introduction

Implantation requires highly complex and orchestrated interactions between the maternal endometrium and the newly formed embryo (Yoshinaga, 2008; Harduf et al., 2009). Compelling clinical evidence demonstrates an association between abnormal implantation and reproductive failure, both in the natural setting as well as following use of assisted reproductive techniques (Horcajadas et al., 2004; Mirkin et al., 2004, 2005; Lea and Sandra, 2007; Yoshinaga, 2008). A yet unknown proportion of implantation failure in IVF might be due to yet undiscovered endometrial defects (Horcajadas et al., 2007, 2008; Aghajanova et al., 2008; Oehninger, 2008). But so far, neither the key molecules involved, nor their strict localization and temporal variations are well known and therefore, the possibility for clinical intervention is limited (Dey et al., 2004; Yoshinaga, 2008).

Studies focusing on early human implantation have been largely descriptive. The amount of information derived from a handful of human in vivo studies is very limited, mainly due to ethical reasons, poor reproducibility and lack of capacity for manipulation/intervention. Functional studies using animals, on the other hand, particularly using the murine model, suggest that implantation is mediated via the coordination of numerous and reciprocal signaling pathways between the endometrium and the embryo (Wang and Dey, 2006; Mardon et al., 2007; Franchi et al., 2009). Murine models can be highly informative, but knowledge gained from them may not necessarily be translated and applied to human implantation. (Lee and De

† Equal contributions.
Mayo, 2004). Although there is an increasing body of knowledge resulting from studies on non-human primates (Fazleabas et al., 2004; Einspanier et al., 2006), such studies are time-consuming, expensive, and it is not practical to obtain large numbers of early implantation stages in these species since their fertility is relatively low. Consequently, there is a fundamental need to develop in vitro models to study human implantation.

Here, we aimed to (i) establish and characterize a three-dimensional (3D) endometrial cell culture in vitro system consisting of both endometrial epithelial and stromal cells; and (ii) to develop an assay to examine the attachment of trophoblast-like cells to the culture. The main difference from previous 3D culture systems reported in the literature (Lalitkumar et al., 2007; Mardon et al., 2007; Teklenburg and Macklon, 2009), is the use of human plasma as a provider of a fibrin matrix. The fibrin gel acts as a stromal matrix and scaffold for cells, and has the advantages of low cost, easy availability and good tolerance to cells (Alaminos et al., 2006). Another difference is the comparison of the behavior of the 3D culture constructed with primary cultures versus established cell lines. Therefore, the major goal of these studies was to develop a 3D culture system that imitates the architecture and physiology of the human endometrium, which could then allow examination of paracrine cross-talk between epithelial and stromal cells, and characterization of the interactions between the trophoblast and the endometrial cells.

Materials and Methods

Tissue collection and cell separation

This study has been approved by the Ethics Committee of the Instituto Valenciano de Infertilidad, Valencia, Spain, the institution in which the endometrial biopsies were obtained and processed for culture of primary endometrial cells, and by the Institutional Biosafety Committee at Eastern Virginia Medical School, where experiments with established cells lines were performed. Written informed consent was obtained from all patients. Endometrial samples were collected from healthy fertile cycling volunteers (oocyte donors) aged 23–39 years, on the day of oocyte retrieval, following standard procedures and ovarian stimulation protocols (Horcajadas et al., 2008). Endometrial samples were minced into small pieces of <1 mm, and then subjected to mild collagenase digestion. Endometrial epithelial cell (EEC) and endometrial stromal cell (ESC) were isolated as previously described (Mercader et al., 2006). EECs and ESCs were cultured until confluence in 75 ml flasks (Nunc, Labclinics, Madrid, Spain).

Construction of the 3D endometrial cell culture using human endometrial cells derived from primary cultures

Approximately 1.0 × 10^6 of cultured primary ESCs were added to a volume of 32.7 μl of 3D culture medium, and then mixed with 400 μl of prepared human plasma (frozen human plasma stored at −80°C quickly thawed in a bath at 37°C). After mixing, 200 μl of tranexamic acid (Amchafibrin, Rotthpharm, Monza, Italy) were added to prevent degradation of the scaffold by fibrinolysis. Rapidly, 38.5 μl of CaCl_2 100 mM were added to induce fibrin polymerization. Finally, 25 μl of agarose 2% (w/v) (Agarose type VII A9045-25G, Sigma, Madrid, Spain) at 45°C in 1 l of phosphate-buffered saline (PBS) was added to get a final concentration of 0.1%. The mixture was immediately transferred to a 12 mm insert (0.4 μm pore size, Corning Inc., Corning, NY, USA), and then the insert was placed inside a culture well (of a 4-, 6- or 12-well cell culture plate, 1.9 cm²/well, Nunc, NY, USA) and allowed to solidify at 37°C (typically not >20 min), and after 20 min the 3D culture medium was added. This medium was prepared by mixing 500 ml of Dulbecco’s modified Eagle’s medium (DMEM) with 5 ml of sodium pyruvate 100 mM (S8636, Sigma). After solidification, 3.0 × 10^5/cm² of primary EEC was seeded onto the 3D matrix of ESC. The endometrial 3D culture model optimized final composition is presented in Table I. This protocol is a modification of the protocol for 3D culture of rabbit conea published by Alaminos et al. (2006).

Construction of the 3D endometrial culture using human endometrial cells derived from established cell lines

The human endometrial adenocarcinoma cell line (HEC)-1A and the immortalized human endometrial stromal cells line (HESCs) (originated from normal endometrium) (Krikun et al., 2004) were purchased from the American Type Culture Collection (ATCC, USA). The Ishikawa cell line (also derived from human endometrial adenocarcinoma cells) was generously provided by Dr Lockwood, from Yale University (New Haven, CT, USA). These cell lines were cultured in vitro according to the manufacturers’ instructions and as published previously (Nishida, 2002; Navarro et al., 2003; Bocca and Archer, 2005; Uchida et al., 2005).

The HEC-1A cells were cultured in McCoy’s 5A medium (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum (FCS, Sigma, St. Louis, MO, USA), 100 U penicillin and 100 μg/ml streptomycin. T HESCs were cultured in DMEM Ham’s F-12 (Sigma) supplemented with 10% charcoal stripped FCS (Atlanta Biologicals, Lawrenceville, GA, USA), 1% insulin, transferrin and selenium (BD, Bedford, MA, USA), 1 mM sodium pyruvate (Gibco), 100 U/ml penicillin and 100 μg/ml of streptomycin (Gibco). Ishikawa cells were cultured in DMEM/F12 supplemented with 10% (v/v) FCS, 200 nM L-glutamine (Gibco) and 100 U/ml penicillin and 100 μg/ml of streptomycin (Gibco). Cells were routinely cultured at 37°C in a humid atmosphere with 5% CO₂. Media were changed every second day.

Initial studies were conducted to determine the optimal composition to result in a 3D culture that could maintain its architecture and viability for at least a period of 10 days in culture after set up and construct of the stromal matrix and epithelial monolayer. A variety of conditions were tested, including among others, the concentration of CaCl₂ that allowed for a rapid solidification time, and the number of stromal cells residing in the matrix. It was shown that with an increase in the stromal cell concentration, the needed CaCl₂ concentration declined. Therefore, the final ‘formula’ used in all experiments involved the use of 2 × 10^6 stromal cells added into 3D matrix. The final construct of the 3D culture system was as follows (Table I): 2.0 × 10^6 stromal cells were suspended in 33.5 μl of medium, and then added into 413 μl of plasma. To prevent degradation of the scaffold by fibrinolysis, the mixture was supplemented with 12.5 μl of 50 mg/ml tranexamic acid (Sigma). At the same time, type VII agarose (Sigma) was melted and dissolved in 1× PBS (Gibco). Thirty microlitres of 1.5% VII agarose also was added into the fibrin. Then, 12.5 μl of 100 mM CaCl₂ (Sigma) was added into mixture. Finally, the mixture was immediately transferred to a 12 mm insert (Corning), and then the insert was placed inside a culture well (of a 4-, 6- or 12-well cell culture plate, Nunc) and allowed to solidify at 37°C. After 20 min, 0.5 ml DMEM/F12 medium was added into the well. Twenty-four hours after the 3D matrix had solidified, 4 × 10^5/cm² human EECs (in 0.5 ml medium) were seeded on the top of 3D matrix and co-cultured in DMEM/F12 medium for 2–3 days, for use in jar spheroid attachment.
experiments; co-cultures were kept for a maximum of 10 extra days to determine long-term viability and functionality (see below).

**Immunohistochemistry**

Immunohistochemistry was conducted to detect vimentin\(^+\) and cytokeratin\(^+\) cells in the 3D cell culture system. Antibodies against vimentin and cytokeratin (Abcam, Las Vegas, NV, USA) were diluted to 1:75. In brief, the 3D culture was fixed with formalin, embedded in paraffin, sectioned into 5–7 \(\mu\)m slices, then deparaffinized with xylene (Fisher, Fair Lawn, NJ, USA) and rehydrated by gradient of ethanol solutions. Antigen retrieval was performed using 10 mM sodium citrate (pH 6) by microwaving. Endogenous peroxidase (Fisher, Fair Lawn, NJ, USA) was abolished by 2% \(\text{H}_2\text{O}_2\) in PBS for 10 min. Non-specific staining was minimized by pre-incubation of sections with 2% goat serum for 30 min at room temperature. The antibodies were incubated for 30 min at room temperature in a humidifying chamber. The secondary antibody [labeled polymer-horse-radish peroxidise (HRP), anti-mouse] was from Dako (Carpinteria, CA, USA). The staining was developed using the 3,3´-diaminobenzidine (DAB) chromogen that was supplied with the Dako kit for 5–10 min at room temperature (Franchi et al., 2008). Sections were counterstained by hematoxylin (Richard Allan, Kalamazoo, MI, USA) and rehydrated by gradient of ethanol solutions.

Measurement of prolactin secretion in the 3D culture system

Supernatants from 3D cultures were assayed for prolactin concentration up to 8 days in culture. Culture medium was changed every other day, and fluids kept at \(-20^\circ\)C until tested. The secretion of prolactin (by the stromal cells and present in the supernatants) was measured with a solid-phase two-site chemiluminescent immunoassay with the Immulite prolactin kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The inter-assay and intra-assay coefficients of variation were 3.2 and 1.9%, respectively, and the limit of sensitivity was 0.5 ng/ml.

**Jar spheroids culture, viability, immunohistochemistry for hCG and secretion of hCG into medium**

**Jar spheroids culture**

The human choriocarcinoma Jar cell line was purchased from ATCC, USA. The Jar cells were cultured in RPMI 1640 (Gibco) with 10% FCS, and 100 U/ml penicillin and 100 \(\mu\)g/ml of streptomycin (Gibco). After reaching 80% confluence, the Jar cells were detached with 0.05% trypsin (Gibco). A single-cell suspension was transferred into an agarose-coated petri dish, and cultured in the petri dish for 5 days to form small spheroids (White et al., 1988). Then, these small spheroids were transferred into an Erlenmeyer flask, and cultured for a minimum of 5 days. The Erlenmeyer flask was kept on a shaker at 110 rpm. All cultures were conducted in an incubator with 5% \(\text{CO}_2\) at 37°C. Jar spheroid size was measured using an optic microscope equipped with a calibrated eyepiece reticule.

In initial experiments to optimize the kinetics of spheroid growth, we investigated the optimal concentrations of agarose resulting in high spheroid suspension rates, and secondly, we investigated the growth curve of Jar spheroids measuring the mean diameter of spheroids up to 20 day in culture (data not shown). Results showed that Jar spheroids of 150–200 \(\mu\)m developed between 5 and 7 days in culture on 3% agarose plus culture medium, at an initial Jar cell concentration of 8 × 10\(^5\)/10 ml medium.

**Jar spheroids viability**

We also performed immunofluorescence experiments using confocal microscopy with a double stain viability assay with calcein (green/live cells) and ethidium bromide (EtBr, red/dead cells) to assess the viability of Jar cells in the spheroids (Invitrogen, Carlsbad, CA). Calcein acetoxy-methyl ester (AM) is a cell-permeant dye that can be used to determine cell viability in most eukaryotic cells. In live cells the non-fluorescent calcein AM is converted to green-fluorescent calcein, after acetoxy-methyl esterification.

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**Table 1** Optimized final composition of endometrial cell culture 3D models: comparison between model using primary culture cells and model with established cell lines.

<table>
<thead>
<tr>
<th>medium</th>
<th>Plasma</th>
<th>Tranexamic acid</th>
<th>CaCl(_2)</th>
<th>VII agarose</th>
<th>Stromal cells</th>
<th>Epithelial cells</th>
<th>Final volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary culture model</td>
<td>32.7 (\mu)l</td>
<td>400 (\mu)l</td>
<td>3.8 (\mu)l (100 mg/ml)</td>
<td>38.5 (\mu)l (100 mM)</td>
<td>Fc:</td>
<td>25 (\mu)l (2%) Fc: 0.1% ((w/v))</td>
<td>1.0 × 10(^5)</td>
</tr>
<tr>
<td>Established cell line model</td>
<td>33.5 (\mu)l</td>
<td>413 (\mu)l</td>
<td>7.5 (\mu)l (50 mg/ml)</td>
<td>12.5 (\mu)l (100 mM)</td>
<td>Fc:</td>
<td>33.5 (\mu)l (1.5%) Fc: 0.1% ((w/v))</td>
<td>2.0 × 10(^5)</td>
</tr>
</tbody>
</table>
ester hydrolysis by intracellular esterases. Briefly, spheroids were grown by adding \(8 \times 10^5\) cells into 10 ml of Jar medium, placed onto a petri dish coated with 2% agarose and incubated in humidified CO\(_2\) chamber for 5–7 days. Spheroids were dual labeled with a final concentration of 5 \(\mu\)M calcein AM (Invitrogen) and 1 \(\mu\)M EtBr (Sigma). Spheroids were incubated at room temperature for 15 min then kept on ice to stop the reaction. A positive control was established by adding isopropanol (70% isopropyl alcohol, Sigma) to one sample to purposely compromise membrane integrity thus allowing EtBr to freely enter the cell. The labeled spheroids were protected from light, then placed into an eight-chamber slide and imaged on the Zeiss LSM-510 laser confocal microscope (Zeiss, Jena, Germany). The argon laser at 488 nm excitation wavelength was used to detect calcein (viable cells) and the HeNe1 laser at excitation wavelength 543 nm wavelength was used to detect the EtBr (dead cells). The multitrack setting was used to gather dual labeling simultaneously for each spheroid. Lasers were optimized and images obtained with use of the Z stack software.

**Immunohistochemistry**

Briefly, Jar spheroids of 150–200 \(\mu\)m were transferred into chamber slides and cultured for 24 h. The slides were fixed using pre-chilled methanol (at \(-20\) C), and kept in \(-20\) C for 10 min. Jar spheroids were incubated overnight at 4 °C with the primary antibody. An antibody against \(\beta\)-hCG (Abcam) was diluted 1:25, and the secondary antibody (labeled polymer-HRP, anti-mouse) was from Dako (Carpinteria, CA, USA). The staining was developed using the DAB chromogen that was supplied with the Dako kit for 5–10 min at room temperature. After ethanol and xylene fixation images were obtained.

**Measurement of \(\beta\)-hCG secretion**

Jar spheroids of different sizes were inoculated into different wells in a 24-well plate. After 48 h of culture, supernatants were collected, and stored at \(-80\) C until assayed. The secretion of \(\beta\)-hCG by Jar spheroids was measured using a solid-phase two-site chemiluminescent immunometric assay with the Immulite 1000 hCG kit (Siemens, Los Angeles, CA, USA). The inter-assay and intra-assay coefficients of variation were 6.9 and 4.7%, respectively, and the limit of sensitivity was 1.1 mIU/ml.

**Development of an attachment assay for Jar spheroids to the 3D culture system**

The 3D culture system was set up into wells in 4-well plates (Nunc), and cultured for up to 7 days before use. Fifteen Jar spheroids were then transferred onto the top of the 3D culture system one by one with a fine mouth-controlled Pasteur pipette. The diameter of Jar spheroids was between 150 and 200 \(\mu\)m. After the indicated co-culture time, the 4-well plate was transferred into a bucket of a centrifuge (the top facing the bottom of the swinging bucket), and then centrifuged at 1000 rpm for 5 min. The attached Jar spheroids were counted under regular optic microscopy (\(\times 40\)). The attachment rate was calculated for each well as follows: attachment rate equals the ratio of the number of spheroids attached to the number of spheroids seeded. In different experiments, epithelial cells (HEC-1A and Ishikawa) and EEC from primary cultures, were allowed to from monolayers and cultured at 37 °C in a humid atmosphere with 5% CO\(_2\). The attachment of Jar spheroids to the epithelial monolayer was analyzed using the same methods.

**Statistical analysis**

Each experiment was performed in duplicate and independently repeated at least three times on different occasions. Results are presented as mean ± SEM. The correlation between Jar spheroids size and hCG secretion was analyzed using Pearson correlation coefficient. Comparison of attachment rates within the different cell monolayers and the 3D models was carried out with logistic regression analysis (culture time effect). Analysis of variance and Chi-Square analysis were used as appropriate. \(P < 0.05\) was considered statistically significant.

**Results**

**Characterization of the 3D culture using primary human endometrial cells (epithelial and stromal) isolated from endometrial biopsies**

The endometrial-like 3D architecture of the culture system was evaluated histologically under light microscopy staining with hematoxylin–eosin (H&E). EEC formed a tight cell monolayer on the top part of the 3D culture system. ESC incorporated into the fibrin-agarose gel became elongated and spread out, showing that the 3D culture system provided an adequate environment for the growth of cells (Fig. 2A). The examination of the EEC demonstrated that these cells were clearly polarized with the nuclei at the bottom of the artificial epithelium near the extracellular matrix (ECM) (see Fig. 2B) and in contact with ESC (yellow arrows Fig. 2B). The protocol of cell separation of epithelium and stroma is really a protocol of enrichment so that there is a possibility that a remaining population of ‘contaminating’ EEC is present in the stromal fraction. We could observe how these ‘contaminant’ EEC formed spontaneous glands in the ESC matrix during the culture (Fig. 2C1). The epithelial cells of these artificial glands could be observed frequently under mitosis (Fig. 2C2). Stromal and epithelial cell origins and location were confirmed by vimentin and cytokeratin positive immunostaining, respectively, in the 3D culture system. Immunohistochemistry for the epithelial cell marker cytokeratin was positive only for EEC in both surface epithelium and in the observed glands (Fig. 2D). Immunohistochemistry for the stromal marker vimentin was positive for the ESC residing inside the 3D matrix (Fig. 2E).

**Characterization of the 3D endometrial culture using human established cell lines**

The endometrial-like 3D architecture of the culture system was evaluated histologically under light microscopy. Epithelial cells formed a tight cell monolayer on the top part of the 3D culture system. Stromal cells (T HESCs) incorporated into the fibrin-agarose gel became elongated and spread out, showing that the 3D culture system provided an adequate environment for the growth of cells (Fig. 3A and B). HEC-1A cells transformed into columnar cells and appeared to have some degree of polarity (Fig. 3C). The occurrence of cell polarity of HEC-1A cells appears to be associated with seeded cell concentration and culture time (data not shown). On the other hand, Ishikawa cells were not polarized, and had a thinner appearance, in occasions such as flat plates in others more cubical (Fig. 3B and D). In Fig. 3F, Ishikawa cells exhibited a strong tendency for filopodia-like
extensions. Stromal and epithelial cell origins and location were confirmed by vimentin and cytokeratin positive immunostaining, respectively, in the 3D culture system. Immunohistochemistry for the epithelial cell marker cytokeratin was positive only for Ishikawa and HEC-1A cells concentrated on the top of the culture as a monolayer (Fig. 3C and D). Immunohistochemistry for the stromal marker vimentin was exclusively positive for THESCs residing inside the 3D matrix (Fig. 3E and F). Finally, Fig. 3G and H present immunolocation of αvβ3 integrin in the 3D culture. Epithelial cells showed strong immunostaining, while stromal cells were more weakly positive, as typically seen in endometrial tissue (Franchi et al., 2008).

Prolactin measurements from the 3D construct supernatants were as follows: Day 2 of culture, 1.15 ± 0.08 ng/ml; Day 4 of culture, 1.13 ± 0.06 ng/ml; Day 6 of culture, 1.17 ± 0.02 ng/ml and Day 8 of culture, 1.2 ± 0.03 ng/ml (not significant). The structure/architecture of the matrix and epithelial monolayer examined after fixation and H&E staining remain unchanged for the 10-day culture period (data not shown).

Figure 1 Diagrammatic representation of the construction of the 3D endometrial culture system, and co-culture with Jar spheroids. Human plasma, stromal cells, CaCl₂, tranexamic acid and agarose are assembled in a culture well to form the 3D matrix. Solidification of the matrix occurs in 20 min, and the matrix is assembled in a 24-h culture period. Then, epithelial cells are seeded on the top to form a monolayer, and the 3D culture system is established. It takes 2–3 days to form a tight monolayer. Jar spheroids are developed using single Jar cell by spheroid culture technology, in a process that takes 5–7 days of culture. Jar spheroids are then transferred to the top of 3D culture system to initiate attachment experiments.

Jar spheroids: immunohistochemistry for hCG, and secretion of hCG into medium

We assessed Jar spheroids hCG secretion by ELISA. The secretion of HCG was expressed as per spheroid, per 48 h culture period. The amount of hCG secreted into the medium was directly proportional to the spheroid size (Fig. 4A, Pearson correlation coefficient, r = 0.982, P < 0.0001). Jar spheroids were also immunostained with an antibody to β-hCG. Approximately 5–10% cells stained positive for β-hCG (Fig. 4B).

Figure 4C (calcein), D (EtBr) and E (combined-merged calcein—EtBr image) show the confocal microscopy results of experiments designed to examine the viability of Jar spheroids in culture. The viability of the Jar spheroids, 150–200 μm in diameter, grown for 5–7 days in culture was >99% live cells. Figure 4F shows a positive control for EtBr (cells treated with isopropanol) with 100% non-live cells. Figure 4G (calcein) and H (combined-merged calcein—EtBr image) shows a representative Jar spheroid grown for 10 days, with ≏75% live cells.

Attachment rate of Jar spheroids to the 3D culture system

First, we investigated the attachment kinetics of Jar spheroids to endometrial cell monolayers from established cell lines and primary cultures. The attachment of Jar spheroids to the various monolayers gradually and significantly increased with the co-culture time (linear regression analysis, P < 0.0001 for all cell types). Jar spheroids attached to Ishikawa monolayers rapidly. After 1 h co-culture time of Jar spheroids with an endometrial monolayer, the attachment rate of Jar spheroids to the Ishikawa monolayer was significantly higher than to EEC and HEC-1A monolayers (92.8 versus 58.6% and 58.6%, respectively, P < 0.05). A 100% Jar spheroids attachment to the Ishikawa monolayer required 2 h of co-culture to ECC monolayer almost 10 h, whereas to the HEC-1A monolayer it required more than 15 h. These results indicated differential endometrial
monolayer adhesiveness to Jar spheroids that was strongly related to the type and functional characteristics of the endometrial cell lines: Ishikawa monolayer adhesiveness > ECC > HEC-1A monolayer adhesiveness.

Second, we investigated the attachment of Jar spheroids to the 3D culture systems. Figure 5A presents a micrograph of the attachment of a Jar spheroid to a HEC-1A/T HESC 3D culture model (co-culture time 7 h, H&E stained, magnification ×400). The space between Jar spheroid and epithelial cells monolayer is clearly visible, and neighboring cells remain superficially attached, showing the attachment of Jar spheroid occurs at many sites. Note the stromal cells embedded in the matrix. Figure 5B presents a micrograph of the attachment of a Jar spheroid to EEC/ESC primary cell culture model (co-culture time 8 h, H&E stained, magnification ×400). Spheroids were closely attached to 3D culture system. In most of the cases Jar spheroids displaced the mono-layer of EEC at the top of the culture.

Figure 5C shows the time-dependency of the rate of attachment of Jar spheroids to the three kinds of 3D culture systems. The attachment of Jar spheroids to the various 3D models also gradually and significantly increased with the co-culture time (linear regression analysis, \( P < 0.0001 \) for all cell types at 0.5 and 1 h). The attachment rate of Jar spheroids to the HEC-1A 3D culture system was significantly lower than to the Ishikawa 3D culture system, at 0.5 h and at 1 h (32.5 versus 87.5%; 59.5 versus 100%, respectively, both \( P < 0.05 \)). A 100% Jar spheroids attachment to the HEC-1A 3D culture system only needed 1 h (Fig. 5D). These results indicated that the attachment competence of the HEC-1A 3D culture system is lower than the Ishikawa 3D culture system. In the case of the 3D system performed with primary EEC, the attachment assays showed attachment rate intermediate of 48 and 65%, at 0.5 an and 1 h. Three hours and 30 min were necessary for the attachment of the 100% of the spheroids.

The impact of several factors potentially affecting the attachment of Jar spheroids to the 3D constructs was examined at 0.5 h. Figure 6A, presents a comparison of attachment of Jar spheroids to agarose (3%)-coated plastic dishes, glass slides, Ishikawa cell monolayers and a 3D construct of Ishikawa/T HESC. Jar spheroids did not attach to agarose, had minimal attachment to glass slides (8% rate), had significantly increased rates of attachment to Ishikawa monolayers (50% rate) and highest and even more significantly increased attachment to the 3D with Ishikawa/T HESC (80% rate). Figure 6B presents a comparison of attachment of Jar spheroids between monolayers (HEC-1A and Ishikawa cells) and their respective 3D culture systems (HEC-1A/T HESC and Ishikawa/T HESC). Both 3D constructs resulted in significantly higher rates of attachment than their respective monolayers (\( P < 0.01 \)). Figure 6C demonstrates the effect of the presence of stromal cells residing in the matrix on the attachment of Jar spheroids. The 3D constructs with Ishikawa cells and fibrin-agarose matrix but without stromal resulted in a significantly lower attachment rate compared with the ‘complete’ 3D Ishikawa/T HESC constructs (\( P < 0.05 \)).
Discussion

Several experimental in vitro model systems have been developed to mimic the early stages of human embryo implantation that occur in vivo. For example, Matrigel and ECM/stromal cell invasion assays have been used in combination with human trophoblast cell lines or blastocysts (in homologous and heterologous models) to mimic trophoblast attachment and invasion (Lalitkumar et al., 2007; Mardon et al., 2007; Teklenburg and Macklon, 2009). 3D co-cultures of human endometrial cells and blastocysts have also been examined (Bentin-Ley et al., 2000; Park et al., 2003; Punyadeera et al., 2003; Petersen et al., 2005). In other studies, tissue fragments from human first trimester decidua parietalis were put in close contact with spheroids of trophoblast/choriocarcinoma hybrid cells as a model of the invasive trophoblast (Helige et al., 2008). However, mimicking the physiological 3D architecture of the endometrium is clearly a challenge, and an optimized and well characterized 3D model has not yet been reported, and moreover, studies using these 3D models in conjunction with blastocysts/trophoblast cells are very limited.

Based on the previous work of Alaminos et al. (2006), that described the construction of a complete rabbit cornea substitute using a fibrin-agarose scaffold, we developed a 3D endometrial culture model that imitates the normal human endometrium, using both primary cultures of endometrial cells and epithelial/stromal cells from established/immortalized cell lines. The architecture of the 3D constructs remained intact for at least 10 days of culture, and the viability and functional competence of the stromal cells were confirmed by an unchanged secretion of prolactin during an 8-day culture period. Furthermore, the 3D demonstrated intense epithelial cell expression of αvβ3 integrin, a well characterized cell adhesion protein.

The fibrin gel was used as scaffold for maintaining the 3D structure. Fibrin gels have many advantages such as promoting cell migration, proliferation and matrix synthesis through the release of platelet-derived growth factors and the transforming growth factor beta (Sierra and Saltz, 1996). There is the possibility to incorporate growth factors and other bioactive peptides and proteins into the fibrin gel (Hubbell, 1995; Lewis et al., 1997). This would render a more tissue-specific environment for the cells and further amelioration of cell function might be expected as the gel structure also serves as a semi-permeable membrane that separates the cells from direct contact with the medium. Furthermore, the fibrin gel cannot induce toxic degradation. Cells cultured in a 3D environment have been shown to better represent in vivo cellular behavior than cells cultured in a monolayer (Schindler et al., 2006). Here, stromal cell concentration was associated with the solidification of 3D matrix. With an increase of stromal cell concentration, the need for CaCl2 concentration decreased. This indicated that the stromal cells really took part in the process of 3D matrix solidification as an organic component, which appears to differ from other 3D models, such as those using a collagen matrix (Gellersen et al., 2007; Mardon et al., 2007).

Human primary endometrial cells and established cell lines have strengths and weaknesses in this model. The primary endometrial
obtained from cycles where the endometrium was exposed to supraphysiological concentrations of sex steroids, and that these affects might make a difference on the 3D behavior (i.e. cell–cell communications and attachment) when compared with endometrium obtained from natural cycles. Moreover, the availability of large numbers of these cells needs repeated and numerous biopsies of volunteers, isolation of epithelium and stroma and culture, with more difficulties in terms of freezing for storing and sub-culturing purposes. EECs, in particular, cannot be passaged or maintained successfully for long periods of time. On the other hand, it has been shown that human ESCs are particularly amenable to RNA-mediated silencing techniques (Jones et al., 2006).

Conversely, the use of the established epithelial cell lines provides immediate and easy access to already frozen cells (knowledge of passage and genetic modifications/transformations), ample numbers of cells to perform unlimited numbers of experiments and lower cost. Moreover, Cervero et al. (2007) used an in vitro model for embryonic adhesion, composed of a human endometrial cell line (HEC1-A) and B6C3F1 mouse embryos to successfully silence genes.

It is essential to confirm the purity and localization of the different cell types in these models. Consequently, using immunohistochemistry, we confirmed epithelial/stromal origin and location within the 3D architecture. Some antibodies such as anti-vimentin, bind more specifically to the CEM and the 3D-matrix (Fig. 2E). For this reason, we used other antibodies and other techniques (fluorescence) to confirm this point. The images showed how only stromal cells, those embedded in the matrix (white arrows) and those in contact with epithelial cells (yellow arrows) were positive for the fluorescent antibody anti-vimentin (data not shown). Using primary cells, we confirmed optimal polarization of the epithelial monolayer and an adequate distribution and morphology of stromal cells residing within the matrix. The epithelial cells were organized in a polarized columnar epithelium. Occasional glands spontaneously formed. The origin of these glands could be the result of EEC contaminating the stromal fraction. However, it can be also speculated that these glands could be formed by somatic endometrial stem cells differentiated to epithelial cells in culture. The presence of somatic stem cells and their capacity to differentiate to other cell types has been previously demonstrated in endometrial tissue (Cervelló and Simón, 2009). On the other hand, the epithelial cells from the established cell lines were also able to form a monolayer but were not organized in a similar pattern of polarized columnar epithelium, the cells being more columnar (HEC1-A) or flat/cuboid (Ishikawa).

The adhesion rate of different epithelial cell lines has been well documented using monolayers and trophoblast cells (John et al., 1993; Thie et al., 1994, 1996; Martin et al., 2000; Thie and Denkern, 2002). It has been shown that the HEC-1A cell line has poor adhesive properties although it exhibits the well-known pattern of cell-to-cell contacts of polarized epithelial cells with polarized distribution of integrins (Dominguez et al., 2003). As shown by Heneweer et al. (2005), Ishikawa cells represent a good alternative model for processes involved in human embryo implantation. These authors showed that Ishikawa cells grown as monolayers have a uniform size and a cylindrical cell shape and form tight and adherent junctions. E-cadherin and integrin were detected in all regions of the plasma membrane of Ishikawa cells, including their apical membrane. Furthermore, Ishikawa cells are able to polarize in vitro (Ball et al., 1995), and respond to Jar cells’ contact with rearrangement of F-actin and a
Figure 5 Attachment of Jar spheroids to the 3D culture systems. (A) Jar spheroid attaching to the HEC-1A/T HESC 3D culture system (co-culture time 7 h, H&E stained, magnification ×400). Note space between Jar spheroid and HEC-1A monolayer, with displacement of epithelial cells and contact with the underlying matrix at multiple sites. (B) Jar spheroid attaching to the 3D culture system constructed with primary cells (EEC/ESC) (co-culture time 8 h, H&E stained, magnification ×400). (C) The attachment of Jar spheroids to the various 3D models gradually and significantly increased with the co-culture time (*linear regression analysis, *P* < 0.001 for all cell types at 0.5 h and 1 h. (D) The attachment competence of the Ishikawa cells in the 3D culture system was highest. A 100% Jar spheroids attachment to the HEC-1A 3D culture system required 8 h of co-culture, but the attachment to the Ishikawa 3D culture system only needed 1 h, and EEC showed intermediate values.

Figure 6 Analysis of factors affecting the attachment of Jar spheroids at 0.5 h. (A) Comparison of attachment of Jar spheroids to agarose (3%)-coated plastic dishes, glass slides, Ishikawa cell monolayers and a 3D construct of Ishikawa/T HESC. Jar spheroids did not attach to agarose, had minimal attachment to glass slides, and had significantly increased rates of attachment to Ishikawa monolayers, with highest and even more significantly increased attachment to the 3D with Ishikawa/T HESC. (B) Comparison of attachment of Jar spheroids between monolayers (HEC-1A and Ishikawa cells) and the respective 3D culture systems (HEC-1A/T HESC and Ishikawa/T HESC). Both 3D constructs resulted in significantly higher rates of attachment than the monolayers (*P* < 0.01). (C) Effect of the presence of stromal cells residing in the matrix on the attachment of Jar spheroids. The 3D construct with Ishikawa cells and matrix but without stromal resulted in a significantly lower attachment rate (*P* < 0.05).
We used multicellular spheroids of human trophoblastoid Jar cells as a model for blastocyst/trophoblast attachment. We first confirmed that the Jar cells/spheroids in culture showed positive staining for and secreted hCG into the medium. Results confirmed previous observations by White et al. (1988) who reported that only ~25% cells were positive for hCG by immunostaining, consistent with results in primary and transformed trophoblast cells showing that cells of the cytotrophoblastic phenotype are capable of producing hCG to a much lesser extent than syncytiotrophoblastic cells.

Results of the present studies confirmed high- and low-attachment rates to Ishikawa and HEC-1A cells, respectively, both in monolayers and 3D experiments. Furthermore, since the attachment rate was higher for Ishikawa cells in the 3D culture compared with the Ishikawa monolayer, and the presence of a complete 3D structure (Ishikawa/T HESC-matrix) resulted in higher attachment rates than to a 3D with Ishikawa cells and matrix but without stromal cells, we speculate that that the interaction between stromal cells and epithelial cells in the 3D promotes increased trophoblast attachment.

Because of the very limited availability of fresh primary tissue, cell lines provide adequate tools for most functional studies. By careful selection of the most appropriate cell line for the function under study, considerable relevant functional data (not only descriptive) can be obtained that can be subsequently validated in the limited primary tissue available or in animal models (Hannan et al., 2010). The results of the present studies highlight the integrity and reproducibility of the 3D model and its possible relevance to early in vivo human implantation. Since an important drawback of this 3D endometrial culture is the absence of vascular and immune cells, future research is planned to include these cell types in the co-cultures.

Authors’ roles

H.W. performed all the experiments with established cell lines. F.P. performed all the experiments with primary cultures. S.A. assisted with immunofluorescence and ELISA. S.M.-E. performed immunohistochemistry with primary cells. I.H. and J.M.-M. performed experiments with primary cultures. S.B. participated in study design and paper review. S.M. performed the statistical analysis. S.O. principal, co-investigator, designed the experiments, supervised the technical work and participated in writing of the paper. J.A.H. principal, co-investigator, designed the experiments, supervised the technical work and participated in writing of the paper.

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