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An in-vitro model for stromal invasion during implantation of the human blastocyst

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BACKGROUND: Implantation failure is likely to be a major cause of infertility. Studies in mice have identified a number of molecules that are involved in implantation, but the mechanisms of implantation in the human remain unclear, largely due to the lack of models for implantation in the human that provide functional information. METHODS: Human hatched blastocysts were co-cultured with human endometrial stromal cell monolayers. Time-lapse photography of implanting blastocysts, immunostaining for cytokeratin and actin, and measurement of hCG secreted into the culture supernatants were performed. RESULTS: Blastocysts attached to and implanted into the stromal cell layer. Trophoblast outgrowth onto, and invasion into, the stromal cell layer occurred largely at two opposite poles, the orientation of which was aligned to that of the stromal cell fibroblasts. High-resolution image analysis demonstrated that the trophoblast completely penetrated the stromal cell layer. Immunostaining of whole-mounts of implantation sites revealed distinctive actin and cytokeratin-positive anchoring structures adjacent to the basal surface of the trophoblast. Blastocysts implanting into stromal cells secreted higher levels of hCG compared with those cultured on plastic. CONCLUSIONS: A robust model for the study of mechanisms of implantation of the human embryo into the endometrial stroma has been established.

Key words: blastocyst/co-culture/endometrial stroma cells/implantation/trophoblast

Introduction

The high incidence of failure to establish a pregnancy in women undergoing IVF after replacement of high-quality embryos points to the importance of disruption of implantation as a major cause of pregnancy failure in IVF. These observations indicate that impaired implantation, in addition to events such as abnormal embryo development, are important underlying causes of infertility.

Implantation of the human blastocyst is likely to be mediated via a number of signalling and adhesion molecules, although the precise molecular mechanisms involved in the human are not understood. Although the later stages of human implantation in which the trophoblast invades the decidua and maternal vasculature have been widely studied by analyses of first-trimester placental tissue (for a review, see Bischof *et al*., 2000), analysis of the early stages of implantation remains an intractable problem, largely because early implantation sites in the human are inaccessible to experimental manipulation *in vivo*. Most of the literature on human blastocyst development and implantation is essentially descriptive. The development of new treatments to overcome implantation failure and associated infertility will rely upon the identification, in functional studies, of potential molecular targets that have key functions in implantation of the human embryo.

Functional studies using animal models, particularly mice, have revealed a number of molecules that have a function in implantation. Genetic studies using gene knockout methodology have identified a number of growth factors and receptors, including epidermal growth factor (EGF) receptor (Threadgill *et al*., 1995), leukaemia inhibitory factor (LIF) (Stewart *et al*., 1992) and interleukin 11 (IL-11) (Bilinski *et al*., 1998; Robb *et al*., 1998) that have a function in embryo implantation. In addition, in-vitro studies have shown that heparin-binding epidermal growth factor (HB-EGF) mediates the adhesion of mouse blastocysts (Raab *et al*., 1996), and fibronectin and its receptors mediate trophoblast outgrowth in models of blastocyst adhesion (Yelian *et al*., 1995).

Studies in mice have provided clues about which molecules may be involved in human implantation, although the differences in reproductive physiology between the two species

means that knowledge gained from mice does not necessarily translate directly to humans. Primates provide a more physiologically relevant model, and valuable information is emerging from in-vivo and in-vitro studies in baboons (Leach *et al*., 2001; for a review, see Fazleabas *et al*., 1999). Important insights into implantation have also been gleaned from some striking analyses of marmoset early implantation sites (Enders and Lopata, 1999).

Most previous studies in humans are largely confined to a description of the expression profiles of various molecules in the endometrium obtained at different stages of the menstrual cycle. The levels of a number of molecules have been shown to peak during the window of implantation, particularly the extracellular matrix (ECM) receptor integrin $\alpha_v\beta_3$ (Lessey *et al*., 1992), LIF (Cullinan *et al*., 1996) and HB-EGF (Yoo *et al*., 1997; Leach *et al*., 1999). The expression of molecules in human peri-implantation blastocysts is less well studied. Integrins α_3, α_v, β_1, β_4 and β_5 are expressed on early, pre-implantation human embryos (Campbell *et al*., 1995), the EGF receptor (Chia *et al*., 1995) and the HB-EGF receptor Erb B4 (unpublished data), and have also been shown to be present on the trophectoderm of peri-implantation human blastocysts; this indicates that these molecules may have a function in implantation in the human. There are few models of human implantation that have been exploited to dissect the implantation process and from which information about the function of specific molecules involved has been obtained.

Here, a model system has been characterized that will allow the dissection of molecular and cellular mechanisms that underpin implantation. The model involves the co-culture of primary human endometrial stromal cells in a monolayer with human peri-implantation blastocysts. It was shown that blastocysts attach to the stromal cells and the trophectoderm subsequently undergoes outgrowth on, and invasion into, the stromal cell layer, thus penetrating under the stromal cells.

Materials and methods

Endometrial stromal cell culture

Endometrial tissues at different stages of the menstrual cycle were obtained from women aged 20–49 years undergoing hysterectomy for benign indications or sterilization. Patients had a regular 26- to 33-day menstrual cycle, and had received no hormonal medication in the preceding 3 months.

Endometrial stromal cells were isolated with the use of a method described previously (Fernandez-Shaw *et al*., 1992). Briefly, endometrial tissue was cut into small pieces and digested in 330 U/ml collagenase type I (Worthington Biochemical Corporation, New Jersey, USA) in Dulbecco's modified Eagle's medium (DMEM) for 1 h at 37°C. Stromal cells were separated from intact glands by filtration of the digested tissue through a 40 µm gauze (Lockertex, Warrington, UK). The stromal cells in the filtrate were purified by centrifugation at 670 *g* through a 25–60% Percoll step gradient, diluted in phosphate-buffered saline (PBS), pelleted by centrifugation at 300 *g* and resuspended in PBS in 0.1% bovine serum albumin (BSA). The cells were plated into 75 cm^2 tissue culture flasks (10^6 cells per flask) maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 50 IU/ml-50 µg/ml penicillin-streptomycin at 37°C in a humidified environment with 5% CO_2 in air. Stromal cells were used between passages 2 and 10. The stromal cell cultures consistently yielded at least 92% purity as assessed by expression of the stromal cell marker Thy-1. The cells were plated onto 13 mm diameter glass coverslips (Chance Propper Ltd) size 0 for the implantation model experiments.

Embryo collection and culture

Ethical approval for this study was obtained from the Oxfordshire Research Ethics Committee, and a research licence was obtained from the Human Fertilisation and Embryology Authority (HFEA). Embryos were donated for research with informed consent from patients attending the Oxford Fertility Unit, John Radcliffe Hospital, for IVF treatment.

Embryo culture

Ovarian stimulation, oocyte retrieval, insemination and grading of the quality of day 2 embryos were performed as described previously (Dokras *et al*., 1993). Grade A or B embryos donated for research were transferred to 100 µl of a complex serum-free medium (CSFM3) supplemented with 1 mmol/l HB-EGF and 2.5% human serum albumin (Martin *et al*., 1998), and overlaid with 1 ml of light paraffin oil (Sigma Chemical Co., UK). Embryos were maintained in culture, and those that developed to the hatched blastocyst stage (day 6–7) were then transferred onto endometrial stromal cell cultures.

Embryo-endometrial stromal cell co-culture

Hatched blastocysts were cultured on a confluent layer of stromal cells on a 13 mm coverslip in a single well of a 4-well plate. The embryo-stromal cell co-cultures were maintained in 500 µl of pre-equilibrated DMEM up to day 9 post-insemination. The cultures were subjected to time-lapse video microscopy with the use of a Leica DMIRB inverted microscope (Leica, UK) and Photonics Coolview camera (Improvision, UK) in a microscope incubator (Solent Scientific, UK) maintained at 37°C and 5% CO_2. Images were captured every 5 min and processed using Openlab software (both from Improvision). At the end of the culture period the culture medium was collected and stored at –20°C.

Immunohistochemistry

Coverslip co-cultures of embryos and stromal cells were fixed, permeabilized, and stained as described previously (Hotchin *et al*., 1999). The coverslips were blocked using 3% bovine serum albumin in PBS. Cytokeratin and actin were visualized in the co-cultures ($n = 5$) by incubation with 1 in 800 dilution anti-cytokeratin monoclonal antibodies (clone CY-90; Sigma) followed by 9 µg/ml anti-mouse IgG conjugated with fluorescein, and 0.165 µmol/l phalloidin conjugated with Texas Red (Molecular Probes Inc., USA). The coverslips were washed three times in PBS, inverted over Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) on glass microscope slides and sealed with nail varnish. Z-images, taken 0.3 µm apart, were captured and deconvolved with the use of a Leica DMRBE microscope (Leica), a Hamamatsu Orca C4742-95 digital camera and Openlab software (both Improvision).

hCG assays

Secretion of hCG by blastocysts into the culture medium was determined using a solid-phase, two-site fluoroimmunometric assay (Delfia hCG; Wallac, Milton Keynes, UK). The hCG production by each embryo was expressed in mIU per 24 h.

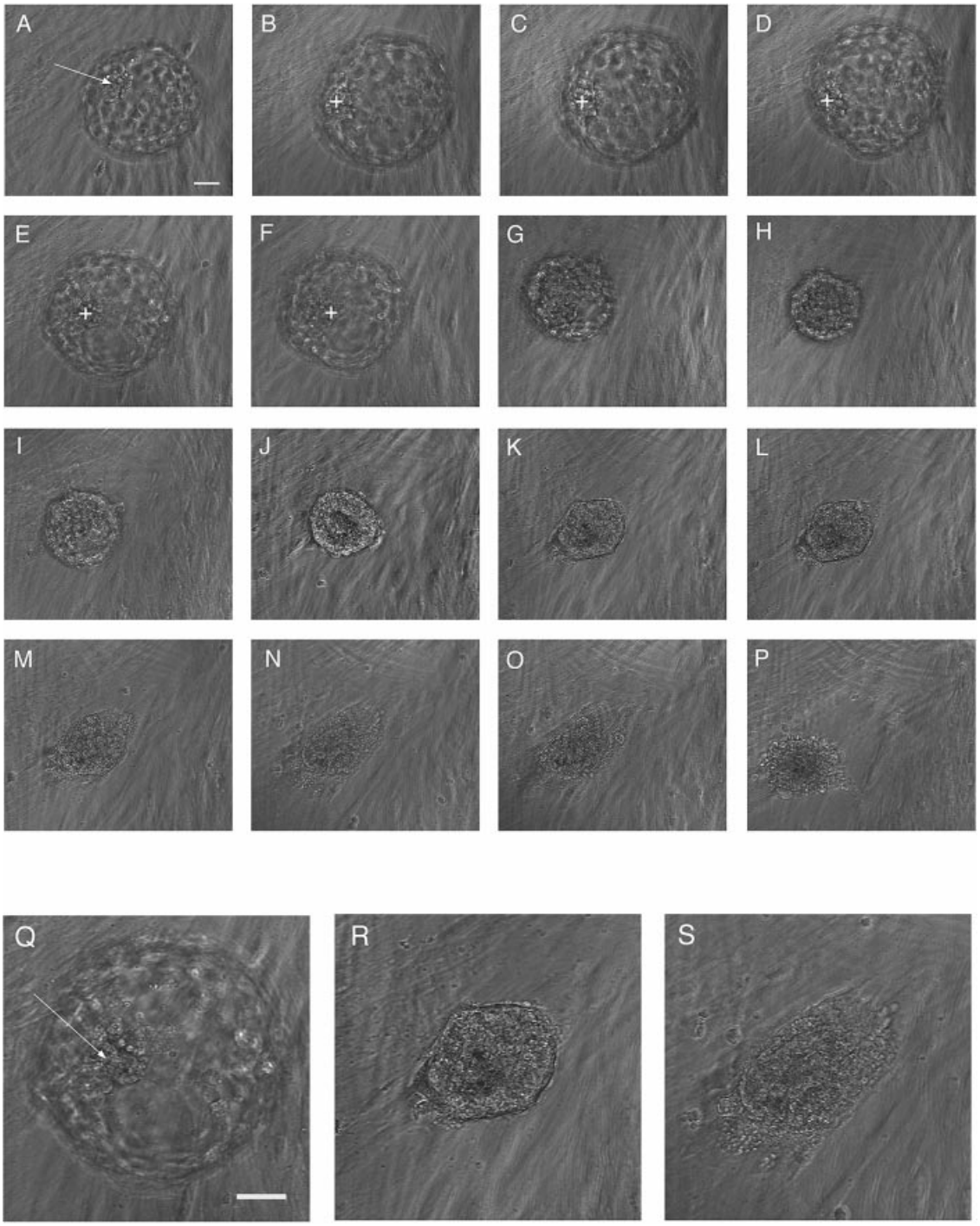

Figure 1. Images captured from a time-lapse video of a day 7 human blastocyst cultured on a layer of human endometrial stromal cells. Time (to the nearest hour) elapsed from the beginning of the co-culture = 0 (**A**); 4.5 (**B**); 5.5 (**C**); 6.5 (**D**); 9 (**E**); 10.5 (**F**); 14 (**G**); 15 (**H**); 21.5 (**I**); 28.5 (**J**); 31.5 (**K**); 32 (**L**); 35 (**M**); 40 (**N**); 42.5 (**O**); and 44 (**P**). The site of adhesion to the stromal cell layer is indicated by the crosses in panels **B**–**F**. Higher magnification of panels **E**, **K** and **N** are shown in panels **Q**, **R** and **S** respectively. The inner cell mass is indicated by the arrows in panels **A** and **Q**. Scale bars = 50 μm.

Results

Human blastocysts attach to and invade endometrial stromal cells

Hatched blastocysts placed on confluent monolayers of human endometrial stromal fibroblasts in DMEM attached to the stromal cells, remained expanded for a period of time and subsequently invaded through the stromal cell layer. Images from a time-lapse recording of a human blastocyst on stromal cells are shown in Figure 1. In these experiments, 31 out of 35 blastocysts attached to the stromal cell layer, and 29 of the 31 exhibited trophoblast spreading and outgrowth onto, and invasion into, the stromal cell layer (Figure 1).

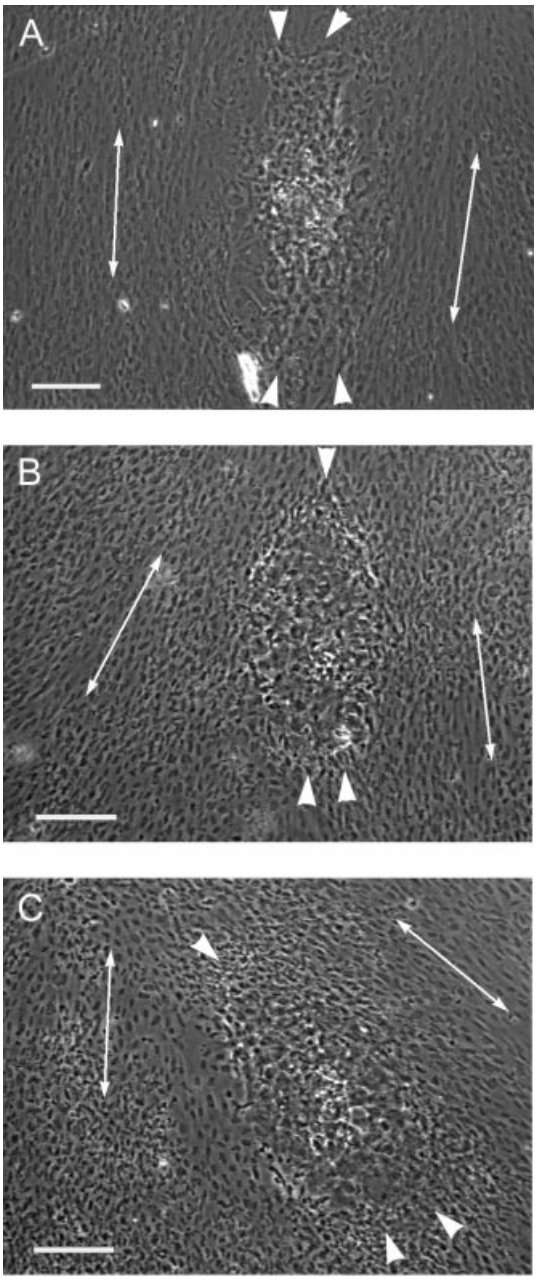

Figure 2. Blastocysts invade stromal cells via two opposite edges and adopt a bipolar form according to the axis of the underlying stromal cells. Phase-contrast images of three blastocysts (panels **A**–**C**) after 48 h in co-culture with endometrial stromal cells. The edges of invading trophoblast are indicated by arrowheads. The long axes of stromal cells aligned in the culture are indicated by arrows. Scale bars = 150 μm.

The blastocysts remained expanded, with the inner cell mass clearly visible, on the surface of the stromal cell layer for a number of hours and appeared to roll on the stromal cells (Figure 1A and B; ~0–5 h). The embryos adhered to the cells within 5–10 h. The inner cell mass was clearly visible in apposition to the stromal cell layer, as shown in Figure 1E, indicating that the blastocysts attached to the stromal cells via the polar trophectoderm. The blastocyst remained anchored to the stromal cells via the trophectoderm adjacent to the inner cell mass for a further period of time. The point of embryo

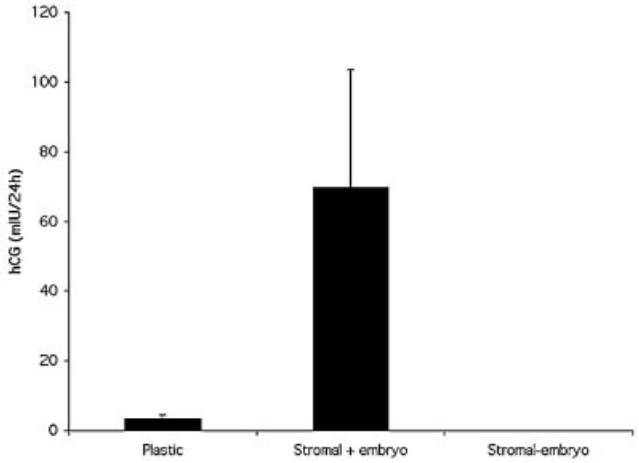

Figure 3. Co-culture of blastocysts on a stromal cell layer induces hCG production. hCG levels in culture supernatants from blastocysts cultured on plastic (n = 23; data from Martin *et al.*, 1998); stromal cells with blastocyst (n = 14), or stromal cells without blastocyst (n = 4) are expressed as mIU per 24 h.

attachment to the stromal cells appeared to be relatively small, since the bulk of the embryo was observed to move relative to the adhesion site (Figure 1B–F; ~5–10 h). The blastocyst then underwent rapid contraction, the inner cell mass became indistinguishable, and the overall diameter of the blastocyst decreased (Figure 1G and H; ~10–15 h). Immediately following this transition the trophoblast protruded from two opposite poles of the blastocyst and underwent outgrowth into the stromal cell monolayer (Figure 1I–O; ~21–44 h, and high magnification at 9, 31.5 and 40 h, Figure 1Q–S). The blastocyst flattened progressively with trophoblast outgrowth on the stromal cell layer (compare Figure 1J and N).

The blastocyst shown in Figure 1 detached from the stromal cells at day 9, after 44 h in culture when the co-culture was disrupted in response to an environmental perturbation (change in pH). The site of blastocyst attachment was clearly visible as an area denuded of stromal cells (Figure 1P).

Implantation into the stromal layer occurs directionally, according to the orientation of the stromal cell fibroblasts

Trophoblast spreading on the stromal cell layer, and invasion through the stromal cells occurred largely in a bipolar fashion, with coordinated projection from two diametrically opposite poles of the blastocyst giving rise to one long and one short axis with lateral trophectoderm penetrating to a lesser extent overall. Examples of three blastocyst implantation sites are shown in Figure 2. The centre of the implanting blastocysts appeared refractile under phase-contrast microscopy, and remained above the stromal cell layer (Figure 2A–C). Elongation of the blastocyst during invasion of the trophectoderm took place in alignment with the stromal cell fibroblasts (Figure 2, white arrows).

Interaction of blastocysts with stromal cells promotes hCG production

The level of hCG in the culture supernatants of blastocyst-stromal cell co-cultures was compared with that produced by

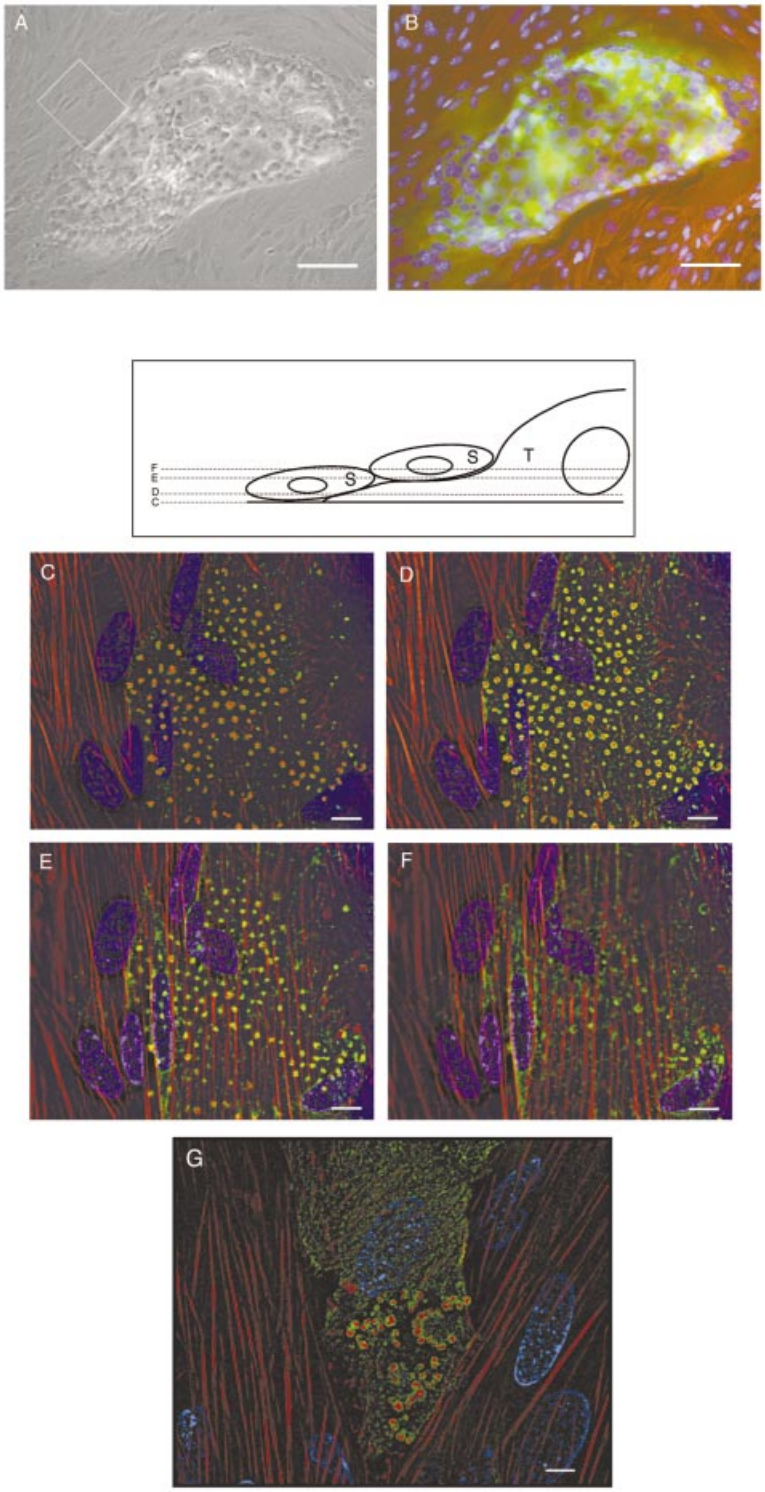

Figure 4. Trophoblast form distinct cell–ECM contacts during implantation into endometrial stromal cells. Phase-contrast image of blastocyst implantation site (**A**) stained for actin (red) and cytokeratin (green) (**B**). Z-images from the area of the blastocyst-stromal cell co-culture shown in **A** and **B** (white square) are represented in the diagram in the middle panel, in which the Z-images shown in **C**, **D**, **E** and **F** are indicated by dashed lines. The relative depths of the Z-images were 0 μm (**C**); 0.9 μm (**D**); 2.7 μm (**E**) and 3.6 μm from just above the surface of the coverslip. Cytokeratin- and actin-positive structures are visible, and cytokeratin filaments appear to protrude from these structures upwards through the cell whilst actin is largely localized adjacent to the membrane. (**G**) A Z-image equivalent to the depth shown in (**C**) of a different blastocyst-stromal cell co-culture, in which actin- and cytokeratin-positive structures can be seen adjacent to the outer edge of the invading trophoblast. S = stromal cell; T = trophoblast. Scale bars: **A** and **B** = 100 μm; **C**–**G** = 10 μm.

blastocysts cultured on plastic (Figure 3). hCG levels in the supernatants of stromal cell-blastocyst co-cultures were more than 10- and 20-fold higher than in supernatants from blastocysts cultured on Matrigel and plastic respectively (Figure 3) (Martin *et al*., 1998).

Trophoblast form distinct cell–ECM contacts during implantation into an endometrial stromal cell monolayer

Blastocyst-stromal cell co-cultures were stained for actin and cytokeratin, and image analyses from two stained embryos are shown in Figure 4. Phase-contrast (Figure 4A) and fluorescent (Figure 4B) images of the embryo-stromal cell whole-mounts revealed that the trophoblast penetrated the stromal cell layer largely at two 'leading edges', and to a lesser extent at the lateral edges of the embryo.

Deconvolution of Z-images obtained from the area outlined in Figure 4A and illustrated in the diagram in Figure 4 was performed to assess the degree of trophoblast invasion into the stromal cell layer. The organization of actin filaments in trophoblast and stromal cells was distinct. Stromal cells had prominent linear actin filaments, aligned along the long axis of the cell, and typical for fibroblasts. Actin staining in trophoblast cells that had invaded and penetrated the stromal cells was localized in a punctate pattern in patches adjacent to the cell membrane, and in short filaments arranged in a mesh, rather than linear filaments, elsewhere in the trophoblast cells (Figure 4C–F). Cytokeratin staining was visible in trophoblast but not stromal cells. Visualization of cytokeratin in the trophoblast revealed prominent ring-shaped structures in the basal region of the trophoblast that co-localized precisely with the punctate actin-positive features so that the blobs of actin were surrounded by rings of cytokeratin (Figure 4C). The cytokeratin-positive trophoblast could be seen adjacent to the coverslip, underlying the stromal cells (Figure 4C). The trophoblast actin and cytokeratin staining was visible until 2.7 μm above the basal surface of the cell (Figure 4D and E). At 3.6 μm the linear actin filaments of the stromal cells could be observed on top of the invasive trophoblast (Figure 4F). The ring-shaped cytokeratin and actin-positive structures described above are shown in a different whole-mount in Figure 4G. These structures were observed toward the outer edge of the invading trophoblast and characteristically occurred in clumps, as shown in Figure 4G.

Discussion

The molecular mechanisms of human implantation remain poorly understood, not least because of the inherent difficulties in studying the process. Here, a model is described for studying mechanisms that mediate human implantation, and which is amenable to experimental manipulation. It has been shown in analyses of the in-vitro implantation sites in this model, that: (i) human hatched blastocysts adhere to, invade and penetrate a cultured endometrial stromal cell monolayer; (ii) trophoblast spreading and invasion occurs largely via bipolar extension of trophoblast processes; and (iii) co-culture of human blastocysts with endometrial stromal cells induces hCG production.

Implantation of the human blastocyst can be described as involving three main stages. First, the priming of the blastocyst and endometrial receptivity are achieved by extensive paracrine cross-talk between the blastocyst and the endometrium. Second, the trophectoderm attaches to the lumenal epithelium of the endometrium by the interaction of adhesion molecules and their receptors, and penetrates the epithelial cell layer and basement membrane. Third, the trophectoderm invades the underlying stroma. The blastocyst-stromal cell co-culture system reported herein represents a robust model for the third stage of implantation of intact human blastocysts.

Three types of model system for the study of human implantation have been reported previously. One involves the attachment of established trophoblast-derived choriocarcinoma cell lines to established cell lines derived from endometrial carcinoma (Thie *et al*., 1995). This model has the advantage that it is relatively straightforward to establish the co-culture because the cells grow rapidly and are uniform. The model has provided some significant data, including the effect of polarity of endometrial carcinoma cells on the adhesion of choriocarcinoma (Thie *et al*., 1996), the strength of adhesive forces between choriocarcinoma cells and endometrial carcinoma (Thie *et al*., 1998), and the function of specific molecules in adhesion (Chervenak and Illsley, 2000). However, the model does not yield information about blastocyst implantation.

A second model system comprises a culture system in which embryos are co-cultured with endometrial epithelial cells, as part of an IVF cycle in which the cultured blastocysts may be returned to the patient (Simon *et al*., 1999) This co-culture model has been used to study the effect of blastocyst-derived factors on the endometrial epithelium. A number of epithelial molecules have been reported to be modulated by the blastocyst, including integrins β_3, α_1 and α_4, the degree of regulation depending upon the developmental stage of the embryo (Simon *et al*., 1997). Leptin secretion is also modulated (Gonzalez *et al*., 2000), and MUC-1, an epithelial glycocalyx component that is thought to inhibit implantation, is diminished on the epithelial cell layer at the site of blastocyst attachment (Meseguer *et al*., 2001). Blastocysts also can induce apoptosis of cultured endometrial epithelial cells (Galan *et al*., 2000). This model thus permits assessment of the first phase of implantation.

A third model for implantation in the human has been established (Bentin-Ley *et al*., 1994) in which human blastocysts are cultured on a three-dimensional structure comprising a reconstructed endometrial stroma and epithelium separated by a basement membrane. This elegant model has the potential to facilitate implantation of the human blastocyst through the two cell layers and effectively allows all three stages of implantation to be visualized (Bentin-Ley *et al*., 2000). However, published reports are as yet limited to the initial attachment to, and penetration of, the endometrial epithelial layer.

In the monolayer system described herein, it is possible to apply high-resolution image analysis to investigate the nature and extent of invasion of the blastocyst into the stromal cell layer. In the present model the blastocysts remain expanded but unattached to the stromal cell layer for a number of hours. After

attaching to the stroma they appear to undergo contraction and become less expanded before entering the invasive stage. The trophoblast appears to have a directionality in that the blastocyst takes on an elongated form as it invades the stromal cells. It appears to have two 'leading edges' of invasion where the trophoblast penetrates the stroma, in addition to the invasive dorsal surface The lateral edges of the embryo protrude and invade the underlying stroma to a lesser extent. The blastocysts undergo elongation along the same axis as the underlying stromal cells. These observations suggest that the trophoblast not only detects, but also responds to, directionality. The blastocyst as a whole acquires a bipolar axis in the same orientation as the underlying stromal cells, indicating that the endometrial stroma may provide specific directional cues for invading trophoblast during implantation. The nature of such cues remains to be determined.

Analyses of Z-images of stained whole-mounts of blastocyst-stromal cell co-cultures revealed that invasive trophoblast completely penetrates the stromal cell layer and appears to establish contact with the underlying stromal ECM. The short, criss-cross pattern of actin filaments is clearly visible beneath the distinctive elongated stromal cell actin filaments. The present analyses also revealed structures containing actin and cytokeratin filaments in the basal region of the trophoblast cells that may have a function, together with other key molecules, in anchoring the cell membrane to the underlying ECM. However, similar structures resembling macrophage podosomes that act as phagocytic foci have been reported in outgrowing mouse trophoblast (Parast *et al*., 2001). The possibility that trophoblast are scavengers of endometrial ECM breakdown products is compelling, and the analyses of ECM receptors and associated molecules that may be present on the trophoblast cell membrane adjacent to these structures will shed light on their precise nature and function.

Secretion of hCG provides an indication of the well-being and developmental status of the blastocyst (Dokras *et al*., 1993). The present data demonstrate that blastocysts implanting into stromal cells secrete high levels of hCG compared with those maintained on plastic or on Matrigel (Martin *et al*., 1998). These data suggest that the implantation model described herein supports prolonged development and well-being of the blastocysts exposed to endometrial stromal cells, thereby implying that the stromal cells provide factors which promote blastocyst development and invasion.

The successful establishment of a pregnancy is influenced by other factors in addition to endometrial receptivity, for example embryo quality. The increased incidence of pregnancies in older women having oocyte donation from younger women indicates that embryo quality reflects genetic integrity. However, the pregnancy rates from donated oocytes approximate to those of fertile, younger women, and the majority of embryos still fail to implant. The ability of the endometrium to facilitate implantation is therefore likely to be a key factor underlying fertility where the embryonic genetic potential is thought to be optimal, as in oocyte donation.

Model systems for the study of complex processes such as implantation can involve different degrees of complexity. It is likely that each stage of the implantation process involves multiple molecular mechanisms; consequently, the more complex the model, the more of these putative mechanisms may be operating within the model. The model presented here will allow the functional dissection of molecules or groups of molecules specifically in the stromal invasion stage of implantation by the addition of specific antagonists or function-blocking agents. In particular, the soluble growth factors and cytokines, and ECM-associated factors that mediate the invasion process can be identified, and their function determined. However, more complex, three-dimensional models are required to dissect further all the stages of early implantation.

The model described here has the potential to be used as an indicator of embryo quality. Currently, the benefits of new treatments or culture conditions for IVF can only be assessed on the basis of embryo morphology or whether the embryos form pregnancies when transferred back to the mother. The availability of in-vitro models such as that presented here will enable embryos to be assessed in terms of their ability to implant, thereby providing a useful measure of embryo quality in response to a particular treatment.

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