Early stages of implantation as revealed by an *in vitro* model

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Abstract

Our limited understanding of the processes underlying steroid hormonal control of human endometrial receptivity is largely due to the lack of a relevant model system. To overcome scarcity of material, we have developed a model in which mouse embryos attach to human Ishikawa cells, which express functional steroid hormone receptors. Blastocysts flushed from day 4 pregnant superovulated mice were transferred to confluent Ishikawa cell monolayers. After 48 h of co-culture, 85% of the blastocysts had attached loosely, but only 40% attached stably to the epithelial cell surface. In contrast, 95% of the embryos attached stably to tissue culture plastic. Thus, weak attachment of a majority of the embryos was followed by stronger adhesion of a smaller proportion. Seventeen percent of the transferred blastocysts modified the epithelial cell surface with loss of MUC1 at the attachment site, extending variably to adjacent epithelial cells. Initially, stable attachment occurred without disruption to the integrity of the epithelial monolayer, but at later stages after the embryo had spread laterally, displacement of subjacent cells was observed. A modest increase in stable attachment, but no changes to MUC1 clearance, was observed after assisted hatching. After 24 h priming of Ishikawa cells by 17 β -oestradiol (OE₂) followed by 72-h incubation with medroxyprogesterone acetate and OE₂, stable attachment increased from 40 to 70%. Initial attachment is efficient either in the presence or in the absence of hormone; steroid treatment increased the incidence of stable attachment. Implantation failure is predicted to occur in this model when embryos fail to progress from initial to stable attachment.

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Introduction

Implantation involves a succession of genetic and cellular signals (Lessey 2002, Aplin & Kimber 2004) that implement a reciprocal interaction mediating apposition and adhesion between trophoblast in the blastocyst and uterine epithelium, followed by trophoblast invasion (Aplin 2006). In the mid-secretory phase of the menstrual cycle, there is a period of receptivity when the embryo can implant (Bergh & Navot 1992, Aplin & Kimber 2004). In both humans and mice, the apposition and adhesion stages are of short duration (Psychoyos 1986, Bergh & Navot 1992, Lopata 1996, Aplin 2000), and both observational and experimental studies have been limited by this constraint. Despite advances in assisted reproductive technology (ART), only 20–25% of the treatment cycles result in a live birth (de los Santos *et al.* 2003). Unravelling the signals that pass between embryo and maternal cells will suggest strategies to reduce implantation failure and increase clinical pregnancy rates in women undergoing ART.

Attachment initially involves the external cell surfaces of trophoblast and uterine epithelial cells over which a glycocalyx is present (Aplin *et al.* 1996, 1997) containing mucins (Ligtenberg *et al.* 1992), which contribute significantly to the anti-adhesive nature of

the apical epithelial surface (Devine & McKenzie 1992, Hilkens *et al.* 1992, Wesseling *et al.* 1996, Lagow *et al.* 1999). Significant upregulation of the transmembrane mucin MUC1 (Hey *et al.* 1994, 1995, Aplin *et al.* 1996, 1998, Meseguer *et al.* 2001) and its associated glycans keratan sulphate (Hoadley *et al.* 1990, Aplin *et al.* 1998), sialyl lewis x (Hey & Aplin 1996) and *Dolichos biflorus* agglutinin (DBA)-binding ligands (Aplin 1991, Jones *et al.* 1998, Campbell *et al.* 2000) occurs during the secretory phase of the menstrual cycle, and might play a role in mediating the initial embryo–uterine epithelial interaction. During embryo attachment, the glycocalyx is reduced in thickness by a locally acting mechanism (Thathiah & Carson 2004, Horne *et al.* 2005). The human blastocyst modifies the glycocalyx of endometrial epithelial cells in co-culture with local loss of MUC1 (Meseguer *et al.* 2001). The molecular mechanism of this phenomenon remains unknown, though selectin–ligand binding has been suggested as one component of the cascade (Genbacev *et al.* 2003). In mice, although MUC1 protein is highly expressed in the luminal and glandular epithelial cells of the uterus in pro-estrous, estrous and metaestrous, at d3 of pregnancy, it decreases in the luminal epithelium, apparently under maternal control, and by d4, it is almost completely lost in both luminal and glandular epithelium (Surveyor *et al.* 1995).

In humans, the blastocyst adhesion stage is of short duration, limiting studies *in vivo*. While *in vitro* studies cannot completely mimic the intrauterine environment, they can model some of the potential interactions (Meseguer *et al.* 2001) and provide a framework within which molecular parameters may be manipulated. Ethical issues and availability limit the use of human embryos and implantation stage endometrial tissue. So, we have turned to Ishikawa, an endometrial epithelial cell line showing moderate apical–basal polarity (Hohn *et al.* 2000, Heneweer *et al.* 2005), steroid hormone receptors (Nishida *et al.* 1985, Hata & Kuramoto 1992, Lessey *et al.* 1996, Mo *et al.* 2006, Uchida *et al.* 2007) and cell adhesion molecules (Castelbaum *et al.* 1997, Somkuti *et al.* 1997, Widra *et al.* 1997), which have been implicated in implantation (Sutherland *et al.* 1993). Although specific mechanics and cellular architecture of implantation vary among species, implantation in mice occurs at a defined window of uterine receptivity (Psychoyos 1986, Yoshinaga 1988), and a reciprocal interaction between the blastocyst and luminal epithelium is essential, leading to interstitial penetration of trophoblast, as in humans. Thus, for modelling implantation *in vitro*, use of mouse blastocysts seemed appropriate to overcome scarcity of human embryos.

We hypothesised that mouse embryos would attach to human endometrial epithelial cell monolayers, that this process might be sensitive to an assisted hatching protocol and to steroidal stimulation, and that attachment might lead to altered mucin distribution at the apical epithelial surface. The observations have led us to formulate new hypotheses regarding the early phases of implantation in humans *in vivo*.

Results

Attachment of mouse blastocysts to Ishikawa cells

In each experiment, embryos from several mice were pooled and randomly allocated to microwells containing confluent Ishikawa cells. In the initial experiment, 21 embryos were transferred and co-cultured for 48 h. Only 10% of the transferred embryos had attached to the cells when the plate was examined at 24 h. At 48 h of undisturbed co-culture, an increase in the number of attached embryos was observed compared with 24 h incubation. Leaving the co-culture for 72 h did not lead to any further increase compared with attachment at 48 h incubation. Hence, in later experiments, we incubated blastocysts with Ishikawa cell monolayers without observation for 48 h to gather data on embryo attachment.

The results presented are the outcome of 18 experiments (Table 1, row 1) that were performed to examine attachment of zona-intact blastocysts naturally hatching in co-culture at 48 h. Subsets of these experiments were used in further analysis for MUC1 clearance/glycocalyx modification, also shown in Table 1. Overall attachment rates are based on mean percentage from each experiment $\pm$ S.E.M. Blastocysts attached loosely (attachment 1) to the cell monolayer at a high rate (85%). Embryos that remained attached after fixation were defined as having attached stably (attachment 2). Of the total transferred embryos, significantly fewer embryos (38%; $P < 0.001$) reached this stage. Attachment sites that were visualised under the phase contrast microscope displayed a complete cell monolayer beneath and adjacent to the embryo attachment site (Fig. 1). Confocal optical sections of stably attached

Table 1 Summary of embryo attachment data.

Cell/embryo treatment	n	N	Attachment 1(%)	Attachment 2(%)	Clearance
Control cells and naturally hatched embryos: overall total	351	18	84.7 $\pm$ 4.6	38.4 ^a $\pm$ 3.3 ($P < 0.001$)	
Control cells and naturally hatched embryos – MUC1 clearance studies (subset of 351 embryos)	154	12	88.8 $\pm$ 4.8	40.0 ^a $\pm$ 5.8 ($P < 0.001$)	16.9 ^b $\pm$ 4.4 ($P < 0.05$)
Control cells on plastic substrate and naturally hatched embryos	48	8	98.9 $\pm$ 1.1	41.3 $\pm$ 9.9	
Control cells on glass substrate and naturally hatched embryos	62	8	0	0	
Controls cells and naturally hatched embryos	99	3	94.0 $\pm$ 3.8	32.8 ^a $\pm$ 5.1 ($P < 0.001$)	
Control cells and assisted hatched embryos (AH)	63	3	93.6 $\pm$ 4.5	53.0 ^c $\pm$ 7.5 ($P < 0.05$)	
Control cells and naturally hatched embryos – for MUC1 clearance (subset from 99 embryos)	49	3	94.0 $\pm$ 3.9	29.2 ^a $\pm$ 7.7 ($P < 0.001$)	11.0 ^b $\pm$ 7.4
Control cells and assisted hatched embryos – for MUC1 clearance (subset from 63 embryos)	33	3	100.00%	57.5 $\pm$ 12.3	12.5 ^d $\pm$ 7.2 (NS)
Control cells and naturally hatching embryos – for steroid studies	48	4	96.9 $\pm$ 1.8	43.3 ^a $\pm$ 5.5 ($P < 0.001$)	
OE ₂ + MPA-treated cells and naturally hatching embryos – for steroid studies	51	4	98.5 $\pm$ 1.5	70.3 ^e $\pm$ 5.7 ($P < 0.01$)	

Statistical comparisons were made as appropriate by one-way ANOVA or Tukey's multiple comparison on arcsine log-transformed data. NS, not significant.

^aAttachment 2 versus attachment 1. ^bClearance versus attachment 2. ^cAttachment 2 in assisted hatching versus attachment 2 in control embryos. ^dClearance by assisted hatching embryos versus clearance in normally hatched embryos. ^eAttachment 2 in steroid-treated cultures versus untreated controls.

Figure 1 Mouse blastocyst attached to Ishikawa cells. Phase contrast image of a mouse blastocyst stably attached to Ishikawa cells in a 96-well microplate after 48 h of co-culture. The embryo is retained at its original location after paraformaldehyde fixation, with trophoblast layer intact. 20× magnification.

blastocysts indicated that stable attachment can occur with minimal or no disruption to the underlying epithelial cell layer. However, when stably attached embryos had begun to spread laterally, displacement or disruption was observed in the epithelial layer subjacent to the attachment site (Fig. 2).

In an attempt to carry out optimal high resolution light microscopic morphological studies and to compare the rates of attachment, Ishikawa cells were grown on culture-grade glass chamber well slides or coverslips as well as on tissue culture-grade plastic. Embryos completely failed to attach on cells grown on glass ($P < 0.001$) compared with cells grown on plastic (Table 1, row 4). As a control, all the transferred blastocysts attached and outgrew efficiently on plastic, while ~60% of the transferred embryos attached directly on the glass substrate.

Clearance of MUC1 from Ishikawa cells occurs after attachment of the mouse blastocyst

Ishikawa cells express MUC1 and associated glycans as a mosaic of varying intensity (Fig. 3A), and their apically specific expression acts as a marker of cell polarity at confluence. From the total of 18 co-culture experiments performed, a subset of embryo attachment sites from 12 experiments was permeabilised and immunostained for MUC1. In a minority of the sites (16.9%), loss of MUC1 was observed from both the epithelial cell surface and intracellular locations with a distinct boundary pattern to the attachment site (Fig. 3B–D and F), while an undisturbed mosaic pattern of MUC1 staining was observed away from the attachment site and also in control cells without co-cultured embryos (Fig. 3A). Figure 3E shows an example of a site from which little perceptible clearance has occurred. The percentage of embryos showing local MUC1 clearance was significantly different from the percentage of embryos reaching attachment 1 and attachment 2 stages ($P < 0.001$ and $P < 0.05$ respectively). The extent of MUC1 clearance from epithelial cells varied, with patches extending radially from ~10–15 cells to ~60–70 cells with patch diameter sometimes reaching ~700 μm (Fig. 3B–D).

Zona pellucida removal increases stable embryo attachment but not local MUC1 clearance

The proportion of embryos attaining initial attachment (attachment 1) from the assisted hatching group (93%) was comparable with naturally hatching blastocysts (94%; Table 1, row 6). However, significantly more embryos (53%) attained stable attachment compared with naturally hatching blastocysts (33%; Table 1, rows 5 and 6).

A subset of assisted and naturally hatched embryo sites was stained with anti-MUC1 antibody. Despite the increase in stable attachment observed after assisted hatching, there was no significant difference in the local clearance of apical MUC1 (Table 1, rows 7 and 8).

Figure 2 Two serial confocal sections of mouse blastocysts attached to Ishikawa cells. Each series runs from the upper z-plane at top left with only the blastocyst visible, to the bottom right lowest plane with clearly visible Ishikawa cell monolayer. In the left half of the figure, a blastocyst with clearly visible intact inner cell mass and trophoblast is attached stably to the epithelial cell layer with minimum displacement or disruption of the underlying cells, marked by an arrow. In the series at right, a laterally spreading mouse embryo is attached stably with displacement of the underlying cells (arrow). At bottom left, the staining indicates uplift of the epithelial monolayer at the edge of the well. DAPI staining.

Figure 3 Surface features of Ishikawa cells in the presence of attached embryos. Indirect immunofluorescence staining for MUC1 with mAb BC2 in paraformaldehyde-fixed Ishikawa cell-embryo co-cultures. Counterstaining with propidium iodide (A–C) or 4',6-diamidino-2-phenylindole (DAPI) (D–F). (A) Control culture with no embryos present. (B–D) Attachment sites with clearance of MUC1 (white bar). Note the variation in the diameter of cleared area. These embryos were displaced during processing, and the position of the attachment site is marked by 'x' in the image. (E, F) Embryos attached on an epithelial monolayer showing less (E) or more (F) clearance of MUC1 beneath and immediately adjacent to the attached embryo. Normal MUC1 expression is observed away from the attachment site.

Steroid hormone treatment of Ishikawa cells increases stable embryo attachment

Expression of oestrogen receptor α (*ER α* or *ESR1*) mRNA in Ishikawa cells was confirmed by RT-qPCR, and progesterone treatment was observed to effect a radical reduction in *ESR1* abundance (data not shown). Cells were grown in 17β -oestradiol (*OE₂*, 10^{-8} M) for 24 h, and were then switched to *OE₂* (10^{-8} M) and medroxyprogesterone acetate (MPA, 10^{-6} M) for 24 h. They were then co-cultured with embryos for a further 48 h in the presence of both steroids. After steroid incubation, increased immunoreactivity was observed for cell surface markers of the progesterone response, including MUC1, keratan sulphate and DBA-binding oligosaccharide (Fig. 4).

Results presented (in Table 1, rows 9–10) are the outcome of four independent experiments with similar number of embryos transferred in both steroid hormone-exposed and non-exposed Ishikawa cells. As shown, after 48 h of co-culture, no significant difference was observed between the two groups in initial attachment. However, a significant increase ($P < 0.01$) was observed in embryos reaching attachment 2 in steroid-treated cells compared with untreated cells.

Experiments ($N=2$) were carried out attempting to define whether the effect of steroid was on the epithelial cells or on the embryos. Cells were exposed to *OE₂* for 24 h, and then to *OE₂* and MPA for a further 24 h. Finally, embryos ($n=18$) were transferred for co-culture in a normal cell culture medium without steroid hormones. There was no significant difference in the percentage of embryos reaching attachment 1 and 2 compared with the control group, suggesting either that the steroid regimen affected both cells and blastocysts, or that a longer steroid hormone exposure may be required to create a more adhesive epithelium.

Transferred embryos are competent to attach

To confirm that blastocysts were viable and attachment competent, in each experiment embryos were incubated in a standard cell culture medium on tissue culture plastic for 48 h. A high percentage of attachment was observed (90–95%), and these sites were all stable to fixation. At 48 h after fixation, indirect immunofluorescence staining with placental lactogen-1 antibody (2 μ g/ml; Chemicon, Harrow, UK) was performed (not shown). Some embryos retained discernible inner cell mass and trophoblast layer, while others were observed to be expanding laterally on the surface with loss of spherical shape and trophoblast spreading across the substrate (not shown).

Expression of sialomucin on Ishikawa cells and secretory endometrium

The sialomucin CD164, which emerged as a candidate adhesion molecule from an informatics screen of cell surface components in endometrium (Aplin & Singh 2008, Singh & Aplin 2009), is expressed in human secretory phase endometrium (d17 of the menstrual cycle) on luminal and glandular epithelial cells, where localisation was prominently apical, and also on the stromal cells (Fig. 5). Ishikawa cells stained homogeneously at the apical surface with antibody to CD164 (Fig. 6). Cell surface localisation was confirmed by flow cytometry of unpermeabilised cells (data not shown). The distribution of CD164 in Ishikawa cells with an attached embryo site was compared to control cells. A confocal z-series of a stably attached embryo (attachment 2) clearly indicates an intact inner cell

Figure 4 Immunofluorescence comparisons of Ishikawa cells treated with OE₂ and MPA versus control untreated cells showing that the steroid regimen increases the expression of MUC1, keratan sulphate (KS) and glycans recognised by the lectin DBA. Green, FITC-conjugated secondary antibody or streptavidin; red, PI.

mass and trophoblast layer and an undisturbed distribution of CD164 beneath and adjacent to the attachment site (Fig. 6). Control and embryo co-cultured Ishikawa cells showed similar homogeneous patterns of staining on the apical cell surface and in intracellular locations.

Discussion

Early morphological studies characterised the process of implantation as having three stages, apposition, attachment and invasion (Enders & Schlafke 1969). Apposition has not been observed *in vivo* in humans (Hertig *et al.* 1959) or other primates (Enders 2000), probably because interactions between the embryo and epithelium are highly tenuous and embryos are lost during processing for histology. In mice, the uterus closes over the embryo to create an implantation chamber wherein apposition stages can be found by serial sectioning (Enders & Schlafke 1969, Fouladi-Nashta *et al.* 2005). Here, the outer trophoblast surface closely approaches the apical uterine epithelium. Attachment of human embryos has been examined on plastic-grown luminal epithelial cells (Meseguer *et al.* 2001) or endometrial biopsies (Bentin-Ley *et al.* 1999, Lalitkumar *et al.* 2007), but the scarcity of human embryos available for these studies means that only limited information could be obtained on the attachment reaction and statistical comparison of different conditions was not obtainable (Lindenberg *et al.* 1989, Bentin-Ley *et al.* 1999).

To study implantation *in vitro*, different types of endometrial and trophoblastic cells have been used, and they have provided valuable information (Hannan *et al.* 2009, Salamonsen *et al.* 2009), but our understanding of this process is still far from complete. The invasion phase has been effectively modelled using embryos adhering and expanding on endometrial

stromal monolayers (Grewal *et al.* 2008), but this ignores the crucial initial phases of attachment and penetration of the epithelium. Human endometrial epithelial cells in primary culture show phenotypic abnormalities and loss of hormone response (Campbell *et al.* 1988, 2000). Ishikawa cells are stable in culture and have functional steroid hormone receptors. An increase in MUC1 in Ishikawa cells after steroid treatment correlates with previous data, indicating that progesterone regulates

Figure 5 CD164 immunofluorescence. Top panels: endometrial biopsies during secretory phase showing strong CD164 staining on the luminal surface (le), glandular epithelial (ge) and stromal cells (s). Bottom panels: negative controls treated with isotype-matched primary antibodies show no staining of either Ishikawa cells (left) or tissue (right).

Figure 6 Confocal serial optical sections of an embryo attachment site stained for CD164. The top left image in the montage is the upper plane view with only the blastocyst visible, while the bottom right is taken in a low plane with clearly visible Ishikawa cell monolayer. At the bottom are higher magnifications of images showing the embryo, apical Ishikawa cell and mid-Ishikawa planes. The stably attached blastocyst has an intact inner cell mass and trophoblast. Apical CD164 is undisturbed adjacent to and beneath the embryo, and a homogeneous pattern of punctate staining is observed. Green, FITC-conjugated secondary antibody; blue, DAPI.

MUC1 expression in endometrial epithelial cells *in vitro* as well as *in vivo* (Kovarík *et al.* 1993). Combined oestrogen–progesterone treatment also increases the expression of glycodefin A and the rate of attachment and adhesion of Jar cell spheroids (Uchida *et al.* 2007). Models that use choriocarcinoma cell spheroids are, however, limited by the absence of a well-polarised outer layer of trophoblast and other phenotypic differences from normal trophoblast.

The present observations of mouse embryos attaching *in vitro* on human cells indicate an initial, clearly visible but highly tenuous interaction that is unstable to shear stresses introduced by medium change or fixation. Conceivably, this might be equated to the *in vivo* stage of apposition, which is reversible, allowing repositioning of the conceptus in the uterus. However, based on the nature of the observations made, we describe this as attachment 1.

In the control experiments, essentially all embryos adhered and grew out on tissue culture plastic as described previously (Glass *et al.* 1980, Morris *et al.* 1983). Mouse embryos also attach efficiently *in vitro* to mouse luminal epithelial cells (unpublished data). Consistent with this, a large majority of the embryos

transferred to human Ishikawa cell monolayers were found to be competent to undergo attachment within 48 h of transfer. These findings suggest that attachment 1 is a phase in which components of the apical glycocalyx may mediate weak binding to receptors on trophoblast (Aplin 2000, Kimber & Spanswick 2000). Less than half of the transferred embryos progressed from this stage to stable (shear stress-resistant) attachment, a figure that echoes the high rates of implantation failure in ART programmes (de los Santos *et al.* 2003, Kovalevsky & Patrizio 2005). Staining at this stage with antibody to the apical glycoprotein CD164 showed no perceptible disturbance to distribution, indicating that interaction with the embryo may not yet have triggered a major reorganisation of the glycocalyx. Given the evident viability of the embryos used in the present study, the data confirm that receptivity is vested in the endometrial epithelial lining, which has the capacity to act as a barrier to the implanting blastocyst (Aplin 2006).

Our data are consistent with previous observations that Ishikawa cells express ESR1. Progesterone down-regulates ESR1 mRNA in Ishikawa cells and similarly during the mid-secretory phase of the menstrual cycle when LE cells are receptive (Lessey *et al.* 1988).

In vitro, stable attachment increased more than 1.7-fold on steroid-treated cells, adding evidence in support of the fidelity of this model of embryo–epithelial interaction. A similar increase in surface receptivity in mouse uterine cell monolayers after hormone treatment *in vitro* has been reported (Lavranos & Seamark 1989). Withdrawal of steroid prior to embryo co-culture reduced the percentage of embryos reaching stable attachment to the control level, so that the increase in attachment in steroid-treated cells could have been due to either an activating effect of steroids on the blastocyst (Paria *et al.* 1998) or the fact that epithelial cells need longer incubation with progesterin to become receptive. Thus, it is not possible to ascertain whether the increase in stable attachment is due to hormone effects on Ishikawa cells or a dual effect on both cells and blastocysts. However, indirect immunofluorescence indicated increases in P-dependent markers including DBA-binding sugars, MUC1 and associated keratan sulphate on Ishikawa cells after steroid treatment. Experiments in steroid-stripped culture medium in various exogenous steroid regimens are now required in order to determine the mechanism of hormone action in this system, and it will be important to investigate any influence on MUC1 clearance that might in turn impact on stable embryo attachment.

Surprisingly, Ishikawa cells grown on glass do not support blastocyst attachment. This result suggests a connection between basal adhesion substrate and apical phenotype, emphasising the possibility that basement membrane may alter polarity and, in turn, receptivity *in vivo*.

In mouse embryo–Ishikawa cell co-cultures, the embryo induces clearance of MUC1 at its attachment site as observed previously in human embryo/human primary epithelial cell system (Meseguer *et al.* 2001). CD164 (Singh & Aplin 2009) acted as a control in these experiments: it is apically distributed and appears undisturbed at sites of stable attachment. Ishikawa cells express MUC1 as a mosaic, whereas mid-secretory luminal epithelial cells *in vivo* show continuous apical immunoreactivity. Despite this limitation, it was readily apparent that cells both beneath and immediately adjacent to attached embryos lacked detectable MUC1, creating a boundary effect of variable diameter, with cells further away in the epithelial layer remaining unaffected. Not all transferred or attaching embryos caused local MUC1 clearance; it is not clear whether this reflects attachment at a later time of co-culture, with clearance following after a delay, or whether there may be intrinsic variations in embryo potency. Thus, although mouse embryos are not normally required to effect clearance *in vivo* since MUC1 is downregulated under maternal control (Surveyor *et al.* 1995), at least some of them retain the ability to do so. Local downregulation of MUC1 is also observed in rabbit implantation chambers (Hoffman *et al.* 1998), and has been postulated to be required in humans (Meseguer *et al.* 2001).

Zona pellucida removal by assisted hatching prior to co-culture increased stable attachment of embryos, indicating a possible effect on embryo activation. The lack of an effect on clearance rates shows that removal of MUC1 is mechanistically independent from stable attachment. However, the present observations indicate that MUC1 clearance is not a prerequisite for stable attachment. Thus, the data are consistent with a dual role of MUC1 as initial docking site and barrier to further advancement of the adhesion cascade (Aplin & Kimber 2004, Aplin 2006) with the involvement of other, higher affinity adhesion systems. Although the current study does not address the nature of embryo-derived signals, removal of mucin appears to be accomplished by direct action of the blastocyst rather than in response to ovarian steroid. There is a possibility of localised cleavage of the extracellular domain of the mucin, because trophoblast in the implanting blastocyst is known to elaborate several proteases (Thathiah *et al.* 2003, Thathiah & Carson 2004), or that short-range soluble signals pass locally between the embryo and maternal cells (Dominguez *et al.* 2002, Cameo *et al.* 2004, Cervero *et al.* 2005).

After the apposition and adhesion phases, there follows displacement/disruption and eventual invasion. Access of the trophoblast to the lateral epithelial surface is a crucial early step in both humans and rodents (Parr *et al.* 1987, Pampfer & Donnay 1999, Lopata *et al.* 2002). In the apposition phase, the presence of a human blastocyst protects epithelial cells against the apoptotic/displacement pathway, while after adhesion it induces apoptosis (Galan *et al.* 2000). After the mouse blastocyst adheres to the Ishikawa cell monolayer, it similarly appears to induce a displacement reaction in cells in close contact, as is the nature of mouse blastocyst implantation. Further work will be required to define the mechanism, which may involve apoptosis.

Though caution is required in extrapolating from data obtained in a model that uses mouse embryos interacting with human cells, the observations reported here are useful in the formulation of hypotheses regarding early implantation events in humans *in vivo*. Our data suggest that most mouse embryos are capable of initiating a dialogue with human uterine epithelium, but that this process is often stalled, as indicated by failure to reach stable attachment within a normal time frame, failure to remodel surface epithelial composition or failure to advance to the stage of epithelial displacement. Previously, the suggestion was made that poor-quality embryos might fail at the epithelial ‘barrier’ phase of implantation (Aplin 1996, 1997). Based on the observation of distinct phases in embryo–epithelial interaction, a hypothesis has been suggested that low-quality embryos undergo initial attachment, but lack the ability to clear MUC1 or advance to stable adhesion or displacement. The model

offers the possibility to dissect molecular mechanisms in embryo attachment, and has the potential to throw new light on implantation and its failure.

Materials and Methods

Animals and superovulation protocol

All work was conducted and licensed under the Animal Act, 1986, and had local ethical approval. CD1 strain of males and MF1 female mice (wild-type outbred; Harlan Olac Ltd, Bicester, UK) were used. Unless otherwise stated, males were caged singly and were <6 months old. All mice were kept under standard environmental conditions of 12 h light:12 h darkness, controlled room temperature (20–22 °C and 40–60% humidity) with food and water provided *ad libitum*.

Female mice were superovulated with 5 IU of pregnant mare serum gonadotrophin (Calbiochem, Nottingham, UK) administered as a 0.1-ml i.p. injection. Ovulation was synchronised by a 0.1-ml i.p. injection of 5 IU human chorionic gonadotrophin (hCG, Intervet UK Ltd, Milton Keynes, UK) 46–48 h later. Females were then placed singly with males of the same strain overnight. The presence of a vaginal plug the following morning (day 1 of pregnancy) was used as an indicator of successful mating.

Retrieval of blastocysts from mouse uteri

Mice at day 4 of pregnancy were killed 94–96 h post hCG administration by cervical dislocation. The uterine horns were dissected, fat was removed and horns were placed directly in flushing medium – Ham's F-10 (Gibco) + 0.4% BSA (Sigma), and ovaries were checked to confirm the presence of corpora lutea. Embryos were flushed from uterine horns using a 28-G sterile hypodermic needle and 1-ml syringe. Predominantly, blastocysts were recovered along with some arrested morulae and fragmented embryos. Embryos were washed through 3 × 60 µl drops of flushing medium followed by two washes in DMEM cell culture medium (Biowhittaker, Cambrex Corp., Cambridge, UK).

For assisted hatching, acidic Tyrode's solution was used. A set of experiments was carried out to examine the effect of zona removal on embryo attachment prior to co-culturing with Ishikawa cells. Approximately 40% of the blastocysts recovered per experiment were transferred to a 60-µl drop of acidic Tyrode's solution (pH 2.3) for 1 min. The resulting zona-free blastocysts were washed in embryo flushing medium (×6) and in DMEM cell culture medium (×3).

Cell culture and transfer of blastocysts to Ishikawa cell monolayers

Ishikawa cells were maintained in DMEM containing 20 mM Hepes (Cambrex) and 10% foetal bovine serum (Gibco) and 1 mM sodium pyruvate (Sigma). In addition to these, the medium was supplemented with antibiotics – 2 mM L-glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin. Cells were grown in microwell plates (Corning Costar, Corning

Ltd, Sunderland, UK) in an atmosphere of 95% air and 5% CO₂. In an attempt to carry out optimal high resolution light microscopic morphological studies, Ishikawa cells were grown on culture-grade glass chamber well slides (Nunc Lab-Tek II, Nalge Ltd, Hereford, UK).

Attachment on Ishikawa cells

Once collected in a final drop of DMEM, embryos were transferred unselectively to confluent Ishikawa cell monolayers grown in a 96-well plate. Between 1 and 5 blastocysts were transferred per well depending upon the total number recovered. Co-cultures were incubated undisturbed at 37 °C in a 5% CO₂ humidified chamber for 48 h, and were then fixed at 48 h in 8% paraformaldehyde (BDH, Poole, UK) in PBS for 30 min at room temperature. Embryo locations were noted before and after fixation. Embryos attached before fixation were defined as having reached attachment 1, while those that remained attached after gentle fixation were defined as having reached attachment 2.

Indirect immunofluorescence

Mid-secretory endometrium was collected with permission from the local ethics committee, snap-frozen and cryosectioned for staining. Fixed attached embryos on epithelial cell monolayers were washed twice in PBS, and (where specified) the cells were permeabilised for 15 min in PBS containing 0.5% Triton X-100 (Sigma). All incubations and washes were carried out at room temperature. The cells were rinsed with PBS (3 × 5 min), incubated with a primary antibody (anti-MUC1 clone BC2, mouse IgG, 0.5 µg/ml, anti-keratan sulphate 5D4, mouse IgG, 0.5 µg/ml or anti-CD164 clone N6B6, mouse IgG, 2.5 µg/ml; BD Biosciences, Oxford, UK) for 2 h, washed in PBS for 3 × 5 min, and then incubated with 1:50 polyclonal rabbit anti-mouse FITC-conjugated secondary antibody (DakoCytomation Ltd, Cambridge, UK) for 1 h in darkness. Antibodies to human MUC1 and CD164 did not cross-react with mice. The cells were incubated with DBA (Sigma; 10 µg/ml), followed by incubation with FITC-streptavidin. The cells were rinsed with PBS (3 × 5 min), and the nuclei were stained with 2 µM propidium iodide (Sigma) or 4',6-diamidino-2-phenylindole dilactate (Sigma) for 10 min. Finally, the cells were rinsed with PBS (3 × 5 min), and the wells were filled with PBS containing anti-fade 1,4-diazabicyclo[2.2.2]octane (Sigma), wrapped in an aluminium foil and stored at 4 °C. Images were captured under phase contrast (Nikon Eclipse TE 300), epifluorescence or laser scanning confocal microscopy using Kalman optics (Olympus microscope connected to a laser scanning attachment (Bio-Rad) and also a Leica SP2 confocal microscope). Z-series optical sections were obtained at 1-µm increments. The laser scan head was operated by standard Zeiss and Bio-Rad software. As a control for non-specific staining by the secondary antibody, staining was performed with omission of the primary antibody or with a negative control antibody of the same isotype (DakoCytomation Ltd, Cambridge, UK). Cells without transferred embryos were also used as controls. Controls (both cultures and tissue sections) were uniformly negative.

Statistical analysis

Blastocysts cultured on Ishikawa cells, culture-grade plastic or culture-grade glass were scored for attachment prior to and after fixation, representing attachment 1 and attachment 2 respectively. Changes induced in the apical glycocalyx in response to attachment were also assessed. Results for overall attachment and MUC1 clearance by naturally and assisted hatched embryos were analysed statistically. Statistical significance ($P < 0.05$) was tested using Student's *t*-test and one-way ANOVA. Normalisation was achieved by taking the arcsine of the square root of the proportion before analysis. Data shown in Table 1 were arrived at by aggregating the means of several experiments in which the total numbers of embryos varied. When data were reanalysed after aggregating the total embryos used (each embryo as a data point), statistically significant differences were conserved in all cases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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