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The role of the osteopontin–integrin αvβ3 interaction at implantation: functional analysis using three different *in vitro* models

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STUDY QUESTION: Does the interaction between integrin and its ligand osteopontin (OPN) mediate embryonic attachment to endometrial epithelium at implantation?

SUMMARY ANSWER: OPN of epithelial origin binds the receptor integrin αvβ3 at the maternal surface to support adhesion during the early stages of implantation.

WHAT IS KNOWN ALREADY: Integrin αvβ3 and OPN are both present in the endometrial luminal epithelium in the mid-secretory phase.

STUDY DESIGN, SIZE, DURATION: Microscopy of attachment sites of blastocysts (mouse, $n = 151$, human, $n = 8$) and OPN- or BSA-coated beads ($n = 488$) interacting with Ishikawa cell monolayers at 24 and 48 h. Levels of epithelial OPN or integrin αvβ3 were altered by siRNA-mediated targeting and the results compared with non-targeting siRNA or mock-transfected controls.

PARTICIPANTS/MATERIALS, SETTING, METHODS: *In vitro* modelling of early implantation with human endometrial cells (Ishikawa) and mouse or human embryos or ligand-coated beads. Immunolocalization of antigen around attached embryos was measured by image analysis with multiple repeats ($n > 3$), allowing a gradient of relative intensity to be detected. Attachment was quantified using a stability scale and protein expression documented by indirect immunofluorescence. Protein associations were probed by pulldown assays.

MAIN RESULTS AND THE ROLE OF CHANCE: Integrin and OPN levels were increased in epithelial cells near to attached embryos. The pulldown assay confirmed OPN-integrin αvβ3 binding ($n > 3$). Decreased attachment stability of mouse embryos observed after siRNA knock-down of integrin αvβ3 or OPN itself, or OPN-coated beads after knock-down of integrin αvβ3, was tested for significance using Kruskal–Wallis with Dunn's *post hoc* tests.

LIMITATIONS, REASONS FOR CAUTION: *In vitro* model. Attachment data using human embryos is limited by embryo availability. Mouse embryo attachment to human cells involves a species crossover so must be interpreted with caution. Ligand-coated beads allow specific molecular interactions mediating attachment to be probed, but obviously lack the adhesion and signaling repertoire of a live embryo.

WIDER IMPLICATIONS OF THE FINDINGS: Some of the literature identifies reduced integrin αvβ3 expression in infertile endometrium; these findings predict that embryo attachment stability will be reduced *in vivo* if integrin levels are low. We suggest that the robustness of the initial attachment of the embryo affects its ability to progress to the post-epithelial phase of implantation; some poorly attached embryos will be lost.

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Introduction

The menstrual cycle-dependent expression of integrin αvβ3 in endometrium was first reported >20 years ago. Maximal levels were observed in the mid-secretory phase (Lessey *et al.*, 1992), suggesting that it might act as a receptor for embryo adhesion at implantation. Many clinical studies have since evaluated αvβ3 expression and its timing in infertility or assisted fertility settings, and there is evidence to correlate reduced or delayed epithelial expression with reduced fertility (Lessey *et al.*, 1994; Lessey *et al.*, 1995), although this has not been consistently observed (Casals *et al.*, 2008; 2012) (further discussed in Lessey, 2011). Introduction of blocking antibodies or arginine-glycine-aspartate (RGD) peptides that target the ligand recognition site of αvβ3 to the uterine cavity of mice and rabbits can reduce the efficiency of embryo implantation. However, since trophectoderm may express this and other integrins, there is no clear consensus on whether such reagents are targeting the embryo, the maternal epithelium or stroma or all three (Sutherland *et al.*, 1993; Schultz and Armant, 1995; Campbell *et al.*, 1995b; Illera *et al.*, 2000; Illera *et al.*, 2003; Rout *et al.*, 2004; Chaen *et al.*, 2012). Mice lacking αv or β3 are fertile, indicating that whatever the role of αvβ3, it can be compensated, at least in this species (Bader *et al.*, 1998; Hodivala-Dilke *et al.*, 1999).

Studies have also focused on identifying a ligand for αvβ3 that is present at the time of implantation. A strong candidate is osteopontin (OPN), a secretory product of the luminal and glandular epithelium (Johnson *et al.*, 2003) the expression of which is elevated in the secretory phase in humans (Apparao *et al.*, 2001; Kao *et al.*, 2002; Borthwick *et al.*, 2003) and at implantation in mice and rabbits (Apparao *et al.*, 2003; Chaen *et al.*, 2012). It is up-regulated by progesterone in human endometrium and is found in secretory bodies beneath the apical surface of epithelial cells (Quenby *et al.*, 2007). In mice, it is up-regulated in the epithelium by nidatory estrogen (Chaen *et al.*, 2012). It is precisely localized to the trophectoderm–uterine epithelial interface in ruminants in which a longer lived adhesive interaction occurs between the two cell surfaces to produce an epitheliochorial interface (Kim *et al.*, 2010). It contains an RGD site that binds αvβ3 and has binding sites for other integrins (reviewed in Johnson *et al.*, 2003). In an estrogen-dependent process, it can activate vinculin-containing adhesion complexes on the embryo surface that recruit extracellular fibronectin (Chaen *et al.*, 2012). Finally, it forms homo-oligomers that might be able to bridge between receptors on trophectoderm and the maternal epithelium. However, mice lacking OPN are broadly fertile (Liaw *et al.*, 1998) and other candidate ligands for αvβ3 (including embryo-derived fibronectin in rats (Kaneko *et al.*, 2013), mice (Schultz and Armant, 1995) and humans (Feinberg *et al.*, 1991) have been put forward.

Implantation remains a bottleneck in assisted reproduction (ART), with high failure rates at this stage. Advances in understanding of the molecular pathways involved have been slow, and direct therapeutic initiatives are almost non-existent. We propose that *in vitro* models will be crucial to identify molecular pathways that might lead to new therapies to improve the rates of implantation of transferred embryos. However, models must be evaluated critically. We and others have reported attachment of human blastocysts to primary epithelial cultures (Bentin-Ley *et al.*, 1999; Meseguer *et al.*, 2001), and others have studied interactions with endometrial stromal (Grewal *et al.*, 2008) or decidual cells (Teklenburg *et al.*, 2010) in efforts to model the post-epithelial phase. Given the practical difficulties associated with access to primary cells and the limited supply of high-quality human embryos, we suggest the need for accessible and convenient models for the examination of the molecular mechanism. Here, we use Ishikawa cells to model the maternal surface, and examine the role of the integrin αvβ3–OPN interaction in the attachment of mouse and human embryos. In addition, we introduce a bead attachment assay that allows analysis of adhesive interactions between the apical epithelial surface and putative embryo surface ligands. We derive from all three models results consistent with a role for OPN and αvβ3 in early implantation.

Materials and Methods

Cell culture

Ishikawa cells, obtained from American Type Cell Culture, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) (both Cambrex, UK), 10% v/v foetal bovine serum (Gibco, UK), 1 mM sodium pyruvate (Sigma, UK), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin. Cells were grown in 95% air and 5% CO_2 on Matrigel (growth factor-reduced, BD Biosciences, UK) coated 14 mm diameter glass coverslips for immunocytochemistry and siRNA transfection optimization, in Matrigel-coated microwell plates (Corning Coster, Corning Ltd, Sunderland, UK) for bead attachment assays and embryo co-culture, and in culture flasks for pulldown experiments. Matrigel was diluted with ice-cold DMEM (1:8 dilution) and left for 30 min at room temperature for coating. Before plating cells, Matrigel-coated wells were washed three times with ice-cold phosphate buffered saline (PBS).

Immunocytochemistry

Ishikawa cells and fixed attached embryos were washed twice in PBS, and, where specified, permeabilized for 15 min in PBS containing 0.05% Triton X-100 (Sigma). All incubations and washes (PBS 3 × 5 min) were carried out at room temperature. Cells were blocked using 100 μl per coverslip of 5% w/v bovine serum albumin (BSA) in PBS for 45 min at room temperature. Cells were incubated with the primary antibodies shown in Supplementary data, Table S1 for 2 h at room temperature. Bound primary antibody was then detected using polyclonal swine anti-rabbit fluorescein isothiocyanate (FITC) (DakoCytomation Ltd, Cambridge, UK), polyclonal goat anti-rabbit immunoglobulin G (IgG) alexafluor 568 (Invitrogen) or polyclonal rabbit anti-mouse IgG FITC (DakoCytomation, UK) as appropriate, all at 1:50 for 1 h. Alternatively, to amplify the detection intensity, biotinylated swine anti-rabbit (1:50) was applied for 1 h and then followed with FITC-conjugated Streptavidin (1:50 DakoCytomation, UK). Coverslips were mounted in Vectashield mountant with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, UK) as a nuclear stain. Images were captured using 10× and oil immersion 40× objectives on a Zeiss AxioObserver inverted microscope (Carl Zeiss, Inc., Europe) with an apotome attachment at 3.12 μm z-increments, or a Leica DMRBE microscope. Images were captured with a Hamamatsu Orca C4742-95 digital camera and analysed with the Openlab software 'Improvision' (Perkin-Elmer, UK).

Western blotting

Thirty-microgram protein was loaded per well on 8% SDS-polyacrylamide gels. The separated proteins were transferred to polyvinylidene fluoride membranes (Millipore, UK) and blocked with 5% BSA in tris-buffered saline/Tween at room temperature for 1.5 h. Membranes were probed with antibodies (see Supplementary data, Table S1) against: integrin αv, integrin β3, OPN or β-actin at 4°C overnight and then washed, incubated with peroxidase-labeled anti-mouse or anti-rabbit IgG secondary antibody

(DAKO) at room temperature for 1 h, washed again, then visualized by enhanced chemiluminescence (Amersham Biosciences, UK). Image J (National Institutes of Health, Bethesda, MD, USA) was used to quantify bands and compare to the loading control.

Immunoprecipitation

Cells at 90% confluence were washed twice with ice-cold PBS and harvested with RIPA buffer without reducing agents. Cell extracts were clarified by centrifugation (1 min, 12 000*g*) and the protein concentration was measured (Bio-Rad). In order to reduce non-specific binding of proteins to protein A-coated Sepharose beads (GE Healthcare, Buckinghamshire, UK), 250 μg of cell lysate protein was incubated with the beads for 20 min at room temperature and the beads removed by centrifugation (20 s, 12 000 *g*. The beads were then added to pre-cleared cell lysates with an optimized volume (15 μl) of primary antibody against integrin αvβ3 or OPN. The tube was sealed with Parafilm and placed on a rotary wheel overnight at 4°C. The next day, beads were pelleted and washed with RIPA buffer (×3) to reduce non-specific binding. Both the supernatant and pellet were mixed with 2× sample buffer, heated for 5 min at 95°C, centrifuged (2 min, 12 000 *g*) then the released protein recovered in the supernatant. Samples were then analysed by western blotting.

Small interfering RNA

Cells at 40–50% confluency were transfected with Dharmafect-2 (Dharmacon) for 48 h using 0–100 nM small interfering RNA (siRNA) (a combination of four siRNA sequences; OnTarget Plus Smartpool, Dharmacon, UK) targeting, integrin alpha v (ITGAV), integrin beta 3 (ITGB3) or OPN in antibiotic-free medium. All siRNA sequences are given in Supplementary data, Table S2. The effect was compared with a similar concentration of non-targeting control (Dharmacon, UK). Cells were analysed by western blotting.

For embryo attachment experiments, cells were transfected with siRNA targeting OPN, ITGAV or ITGB3, or both integrin subunits together, or non-targeting siRNA (100 nM total concentration) using the Dharmafect-2 transfection reagent for 48 h before mouse embryos were transferred. Non-transfected cells (with only transfection reagent or serum-free DMEM-F12) were used for control experiments.

Animals, ovulation induction and embryo recovery

All experiments were conducted and licensed under the Animal Act, 1986, and had local ethical approval for care and use of laboratory animals and standards of humane animal care. Two different strains, CBAB6F1 (Maintained by Biomedical Services, John Radcliffe Hospital, University of Oxford) and C57BL/6 (maintained by the Biomedical Services Unit at the University of Manchester), mice 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 dark and housed in a conventional holding facility at a controlled room temperature (20–22°C and 40–60% humidity) with food and water provided *ad libitum*.

Female mice (6–8 weeks) were superovulated with 5 IU of pregnant mare serum gonadotrophin (Calbiochem, Nottingham, UK) administered as a 0.1 ml intraperitoneal (IP) injection. Ovulation was synchronized by a 0.1 ml IP injection of 5 IU human chorionic gonadotrophin (hCG, Intervert 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. Pregnant mice were sacrificed on Day 2, 48 h after hCG injection. Using sterilized scissors and forceps the lower abdominal cavity was incised and both oviducts dissected following removal of both uterine horns using fine forceps. Two-cell embryos were obtained from the oviduct either by flushing using a 34G blunt-ended stainless steel needle (Cooper Needleworks, Birmingham, UK) and syringe or by mincing using fine forceps under a microscope. Flushed embryos were washed with M2 media (Sigma) supplemented with 4 mg/ml BSA and subsequently transferred into a 60 μl drop of KSOM (Millipore) for further washing and finally cultured in a drop of 30 μl KSOM covered with mineral oil and incubated at 5% CO_2, 37°C until the blastocyst stage. Only expanded blastocysts with a normal morphology observed on Day 5 were included in the study.

Human embryos

Human embryo work was carried out at the Nuffield Department of Obstetrics and Gynaecology, University of Oxford. Ethical approval for this study was obtained from the Oxfordshire Research Ethics Committee and a research license was obtained from the Human Fertilisation and Embryology Authority. Human embryos were donated for research with informed written consent from patients attending the Oxford Fertility Unit, John Radcliffe Hospital. Ovarian stimulation, oocyte retrieval, insemination and grading for Day 2 embryos were performed in accordance with routine clinical procedures. Embryos were assessed according to morphology and those displaying ≥50% blastomere survival were donated for research and were transferred and cultured in Sydney IVF Blastocyst Medium (Cook Medical Ltd) overlaid with 1 ml of mineral oil (Sigma, UK). Embryos were maintained in culture to the hatched blastocyst stage and those selected as Grades A and B in both trophectoderm and inner cell mass were transferred onto epithelial layers.

Embryo attachment assay

Once the Day 5 blastocysts had been collected in a final drop of DMEM, they were transferred unselectively to confluent Ishikawa (untreated and siRNA transfected) cell monolayers in a 96-well plate. Between one and five blastocysts were transferred per well depending upon the total number recovered. Co-cultures were incubated undisturbed at 37°C in a 5% CO_2 atmosphere for 24 h, then for a further 24 h and finally fixed in 4% paraformaldehyde (BDH, Poole, UK) in PBS for 15 min at room temperature. Embryos were examined under an inverted microscope (Leica, UK) and locations were noted before and after fixation. For immunofluorescence imaging, Matrigel-coated coverslips were used in 24-well plates.

After 24 h of co-culture, the stability of embryo attachment was measured. Before the measurement, the plate was shaken three times from side to side. Each measurement was performed manually under the microscope, examining the stability of each mouse embryo upon tapping the stage. Weakly attached embryos visibly vibrate or show tiny lateral displacements upon tapping, but remain in place at the site of attachment. Based on the stability of attachment, scores were assigned to individual embryos: (i) floating; (ii) weakly attached but detached after tapping; (iii) weakly attached and still attached after tapping; (iv) strongly attached and (v) strongly attached and some trophoblast cells observed to be growing out (Supplementary data, Fig. S1).

For the data shown in Fig. 1, the surface intensity of integrin αvβ3 staining was captured from each coverslip and profiled in grey scale. The *X*-axis indicates the distance (pixel) of each image while the Y-axis shows the grey value of integrin αvβ3 expression. Image width was 89 300 pixels; each point on the graph shows the average of intensity of αvβ3 per 5000 pixel. In Fig. 3, each image was also 89 300 pixels in width; each point on the graph shows the average of OPN fluorescence per 10 000 pixel.

Bead attachment assay

Embryo-sized beads (Affi-gel Blue; Bio-Rad; 80–150 μm) were washed five times with PBS and incubated in a 20 μl drop of pre-equilibrated OPN or BSA, respectively, for 2 h at 37°C. Subsequently, 30–50 beads were transferred per well of confluent Ishikawa cells on Matrigel-coated coverslips (for microscopy) or 10–15 beads in the wells of a 96-well plate (for siRNA), and maintained in serum-free DMEM-F12 for 24–48 h, then

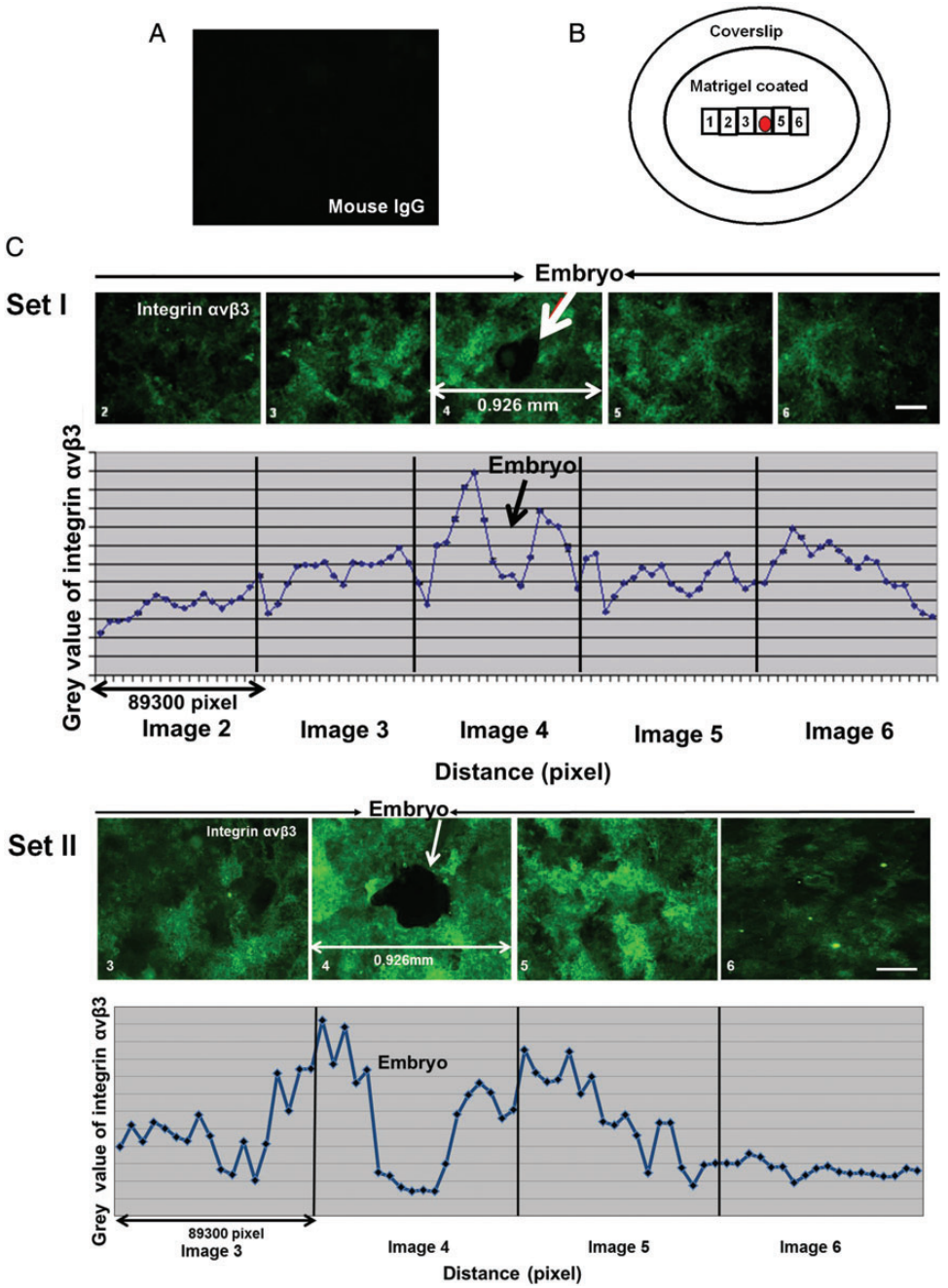

Figure 1 Integrin αvβ3 expression at sites of implantation *in vitro* and the effects of its knock-down by siRNA. (**A**) The first image is a negative control (mouse IgG). (**B**) The locations of the images used in (**C**, Set I) in relation to the site of an attached embryo (red dot) are shown on a diagram of the coverslip. (C, Set II) was based on a similar series of seven images. Images were chosen spanning the areas where intensity changes were evident. (C) Two sets (I and II) of representative images from 31 independent experiments of integrin αvβ3 (antibody LM609) staining on Ishikawa cells co-cultured with mouse embryos. Note the antibody is human specific and therefore does not detect antigen in the embryos. Each attached embryo is located in Image 4 (arrow). After 48 h co-culture, expression of αvβ3 was locally increased adjacent to the mouse embryo, while being unaffected further away from the attachment site, as demonstrated in the surface intensity profile. At higher fluorescence capture efficiency, weaker but significant specific staining is detected in regions away from the embryo (compare Supplementary Fig. S2). Scale bar = 200 μm. (**D**) Two sets (I and II) of representative images of 8 independent experiments of integrin αvβ3 staining on Ishikawa cells co-cultured with single human embryos. Note each embryo (located in Image 4 (arrow)) is strongly positive for the integrin. After 60 h co-culture, expression of αvβ3 was locally increased adjacent to the embryo, while being unaffected further away from the attachment site. This is demonstrated on the surface intensity profile, scale bar = 200 μm.

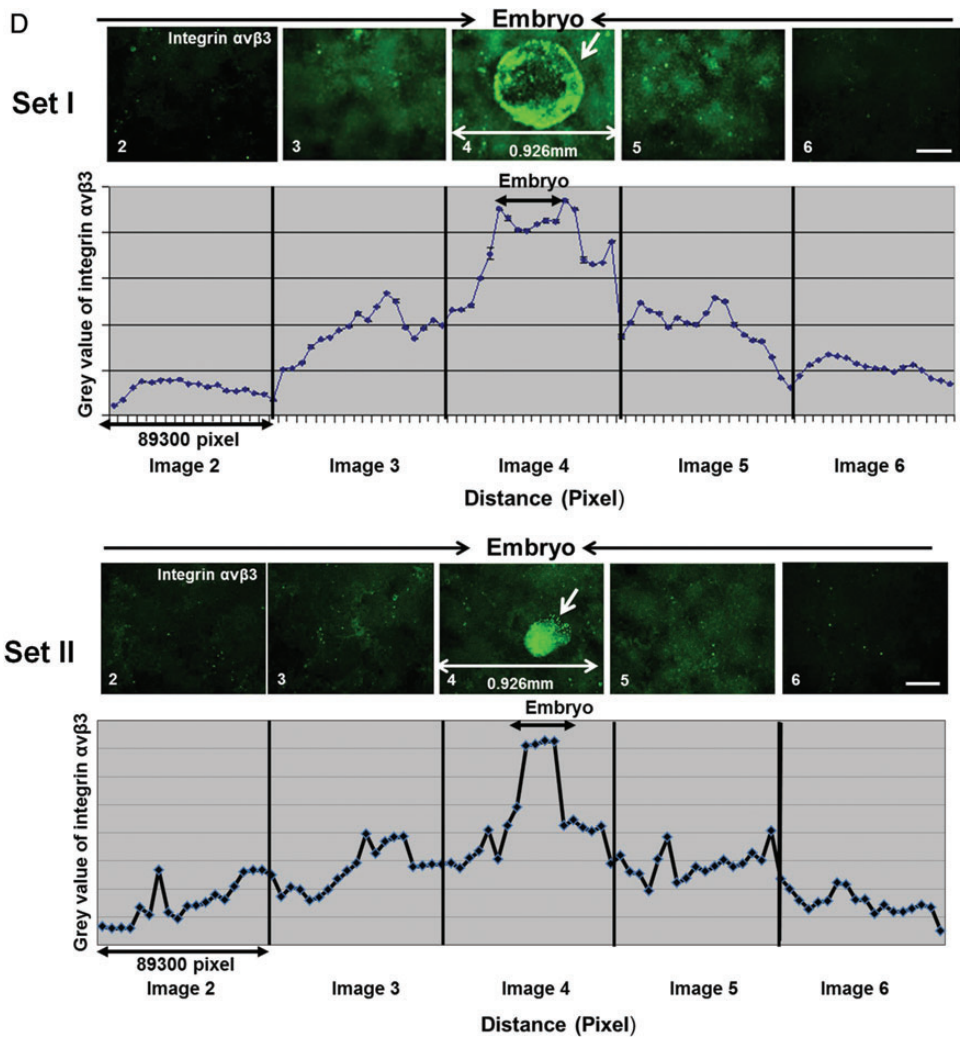

Figure 1 (continued).

washed gently with PBS and fixed with 4% PFA. The number of attached beads were counted and normalized to the total number of beads transferred. The total number of beads used was BSA 240 and OPN 248.

Data analysis

Statistical calculations were done using Prism for Windows (GraphPad, Inc., San Diego, CA, USA). Data are presented as medians. Differences were tested with Kruskal–Wallis tests and with Dunn's multiple comparison tests.

Results

Integrin αvβ3 is locally increased adjacent to attached mouse and human embryos

The presence of the integrin αvβ3 complex was demonstrated by the binding of a well-characterized monoclonal antibody (LM609) to confluent Ishikawa cells. It was detected as a uniform fluorescent signal throughout the monolayer (not shown). Day 5 mouse ($n = 49$; approximately two embryos per well) and Day 6 human ($n = 8$; one embryo per well) blastocysts were transferred to confluent cells and incubated for 48 or 60 h, respectively. Embryos attached stably to the monolayers. Figure 1A is the staining with mouse IgG as a negative control and Fig. 1B is to show the localization of each image including embryo (red spot). The αvβ3 signal was locally elevated at the attachment sites of both mouse (Fig. 1C, Sets I and II) and human (Fig. 1D, Sets I and II) embryos, while being unaffected further away from the attachment site, as revealed by profiling the surface intensity (Fig. 1C and D). Trophectoderm in human embryos was strongly stained by the αvβ3 antibody (Fig. 1D). The antibody does not detect the mouse protein so the mouse embryo is seen as a darker area in images (Fig. 1C, Image 4). Attachment to the apical epithelium was followed by penetration of the embryo with displacement of cells, visible in some sites at 48 h (Supplementary data, Fig. S2). The initial attachment stage prior to epithelial displacement was the focus of subsequent experiments.

Integrin αvβ3 knock-down suppresses mouse embryo attachment

Ishikawa cells were transfected with siRNA targeting integrin αv or β3 siRNA separately or in combination. Various concentrations of four

different sequences that target different regions were used in each case. Western blotting of cell lysates showed significant knock-down by 48 h after transfection (Fig. 2A). Ishikawa cells co-transfected with siRNA targeting both integrin αv and β3 at 100 nM for 48 or 72 h showed ~80% reduction in protein expression compared with control cells. There was no effect on β-actin. However, after 72 h of transfection, alterations were evident in Ishikawa cell morphology (data not shown). Therefore, the duration of transfection used was 48 h.

Day 5 mouse embryos ($n = 68$) were transferred after transfection of Ishikawa cells with siRNA to knock-down integrin αv, β3 or αvβ3, and the stability of attachment was measured after 24 h of co-culture. Embryos on control cells and cells transfected with non-targeting siRNA were significantly more stably attached than embryos co-cultured with cells transfected with integrin αv ($P < 0.05$), β3 ($P < 0.05$) and αvβ3 ($P < 0.001$) siRNA (Fig. 2B). However, after 48 h of co-culture, all embryos had become stably attached.

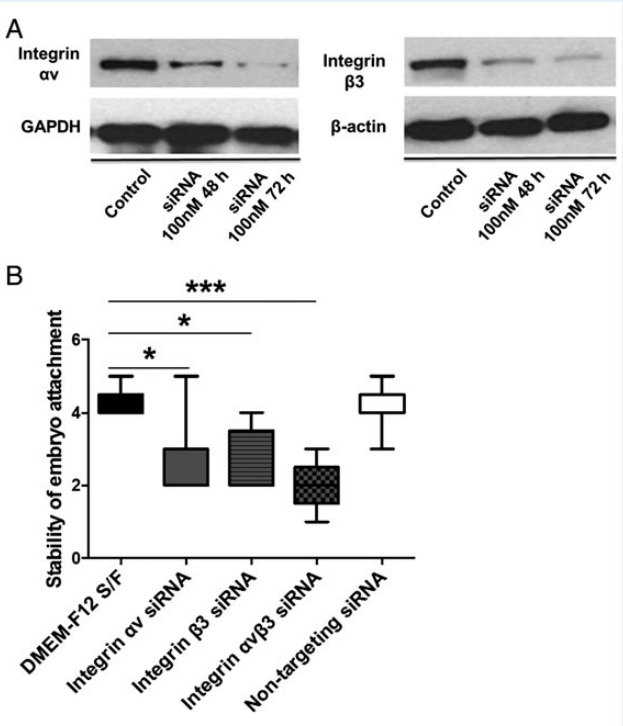

Figure 2 (**A**) Western blots to illustrate knock-down of integrin αv and β3 in Ishikawa cells by 48 h transfection with siRNA at 100 nM. There was an 80% reduction in protein expression compared with control cells. There was no effect on β-actin. (**B**) Day 5 mouse embryos ($n = 68$) were transferred to confluent Ishikawa after 48 h of siRNA transfection and the stability of attachment measured after a further 24 h. Knock-down of integrin αv or β3 or both subunits together. Embryos were significantly more stably attached on control cells (far left bar) and cells transfected with non-targeting siRNA (far right bar) than after knock-down of integrin αv ($P < 0.05$), β3 ($P < 0.05$) or αvβ3 ($P < 0.001$). However, after 48 h of co-culture, all mouse embryos had achieved stable attachment (data not shown). Boxes show quartiles, whiskers show range. *$P < 0.05$, ***$P < 0.001$.

OPN is locally increased adjacent to attached mouse embryos

OPN, a ligand of integrin αvβ3, is present in Ishikawa cells (Apparao *et al.*, 2001; Ramachandran *et al.*, 2013). Mouse embryos were transferred to confluent Ishikawa cells on Matrigel ($n = 6$) and co-cultured for 48 h, during which time attachment occurred. The alteration of OPN distribution in Ishikawa cells near embryo sites was clearly demonstrated by comparing expression in three areas selected by distance. OPN was strongly localized on mouse embryos and adjacent Ishikawa cells, predominantly at the apical cell surface, but also associated with vesicular deposits, while weaker expression was observed in cells further away from the embryo (Fig. 3A, Sets I and II).

To test the hypothesis that OPN acts as a ligand for integrin αvβ3, pull-down assays were carried out from Ishikawa cell lysates. Antibodies against αvβ3 or OPN were used in immunoprecipitation and the resulting blots probed, respectively, with antibody to OPN or the β3 subunit. The results demonstrate that αvβ3 is complexed with OPN (Fig. 3B). The band intensities suggest that only a fraction of the total integrin pool is bound to OPN.

OPN knock-down suppresses mouse embryo attachment

Various concentrations of an siRNA pool containing sequences targeting four different regions of OPN were used to treat Ishikawa cells. Western blotting at various time points showed significant knock-down by 48 and 72 h after transfection. There was no effect on β-actin. However, at 72 h alterations were evident in Ishikawa cell morphology (not shown) consistent with a previous report (Ramachandran *et al.*, 2013). Therefore, the duration of transfection used was 48 h (Fig. 4A).

Day 5 mouse embryos ($n = 28$) were transferred after knock-down and the stability of attachment was measured 24 h later. Embryos on control cells and Ishikawa cells transfected with non-targeting siRNA were significantly more stably attached ($P < 0.0001$) than embryos co-cultured with Ishikawa cells transfected with OPN siRNA (Fig. 4B). However, after 48 h, all embryos were fully attached.

Attachment of OPN-coated beads to Ishikawa cells is an integrin αvβ3-dependent process

Embryo-sized (80–150 μm) microbeads were coated with OPN, then transferred to confluent cells. The beads attached loosely at first and lost their-original positions easily but at 48 h remained stably attached to cells during washing and fixation steps (Fig. 5A).

The number of attached beads was scored against the total number originally transferred (~50 per well). At 24 h of co-culture, 8% of beads bearing BSA remained attached on the gel, while ~30% of beads carrying OPN were stably attached. After 48 h co-culture, 12% of beads bearing BSA were attached on the gel, while ~65% of beads carrying OPN remained stably attached. Beads carrying OPN ($P = 0.0005$) exhibited significantly higher attachment at 48 h (Fig. 5B).

SiRNA-mediated knock-down of integrin αvβ3 suppresses the attachment of beads bearing OPN

Ishikawa cells were transfected with siRNA targeting integrin αv, β3 or αvβ3 for 48 h and beads bearing OPN ($n = 662$) were then transferred.

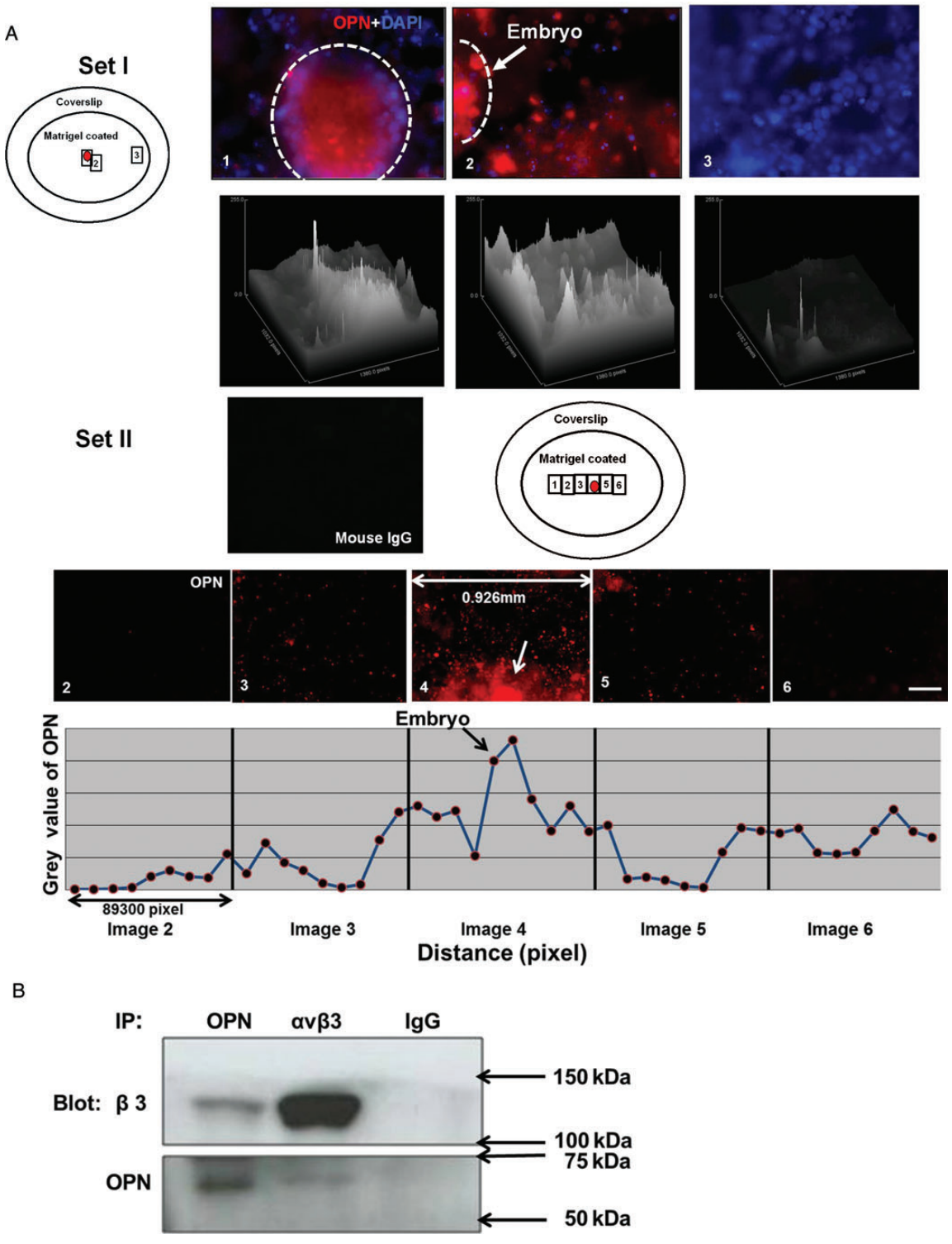

Figure 3 OPN expression at sites of implantation *in vitro* and interaction with integrin αvβ3. (**A**)Set I: a diagram illustrates a coverslip and three areas selected by the distance from the attachment site of mouse embryo (1: at the attachment site, 2: adjacent to the attachment site, 3: away from the embryo). Stronger expression was observed adjacent to the embryo (1 and 2) than in distal areas (3) of the epithelial monolayer. Scale bar = 50 μm. Set II: the first image is a negative control (mouse IgG). The locations of Images 2–6 (from a series of 6) in relation to the site of an attached embryo (red dot, Image 4) are shown in the diagram of the coverslip. After 48 h co-culture, expression of OPN was locally increased adjacent to the mouse embryo, while being unaffected further away from the attachment site, as demonstrated in the surface intensity profile. Scale bar = 200 μm. (**B**) Immunoprecipitates made from Ishikawa cell lysates with antibody to OPN (anti-mouse), integrin αvβ3 (anti-mouse) or mouse IgG were probed for the integrin β3 subunit using a different antibody (anti-rabbit) or OPN (anti-mouse), respectively, to verify the pulldown. These precipitates contained integrin β3 and OPN.

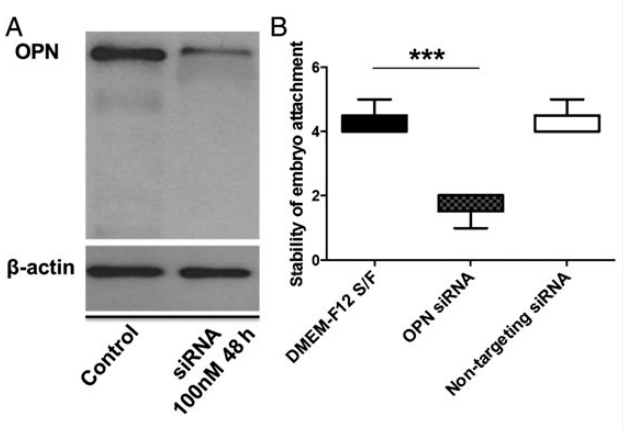

Figure 4 Effects on implantation of OPN knock-down. (**A**) Western blotting analysis showed significant reduction of OPN expression after 48 h of knock-down with siRNA. (**B**) Mouse embryo attachment was monitored after 48 h of knock-down of OPN. After 24 h of co-culture with mouse embryos, embryos were significantly more stably attached on control cells ($P < 0.001$) or cells transfected with non-targeting siRNA ($P < 0.001$) than OPN knock-down cells. Boxes show quartiles, whiskers show range. *$P < 0.05$, ***$P < 0.001$.

Attachment was significantly reduced in each case ($P = 0.0023$) compared with control cells. In both cases, bead attachment on cells treated with non-targeting siRNA ($P < 0.01$) or mock-transfected cells ($P < 0.01$) were similar to the rate on untreated cells in serum-free medium (Fig. 5C).

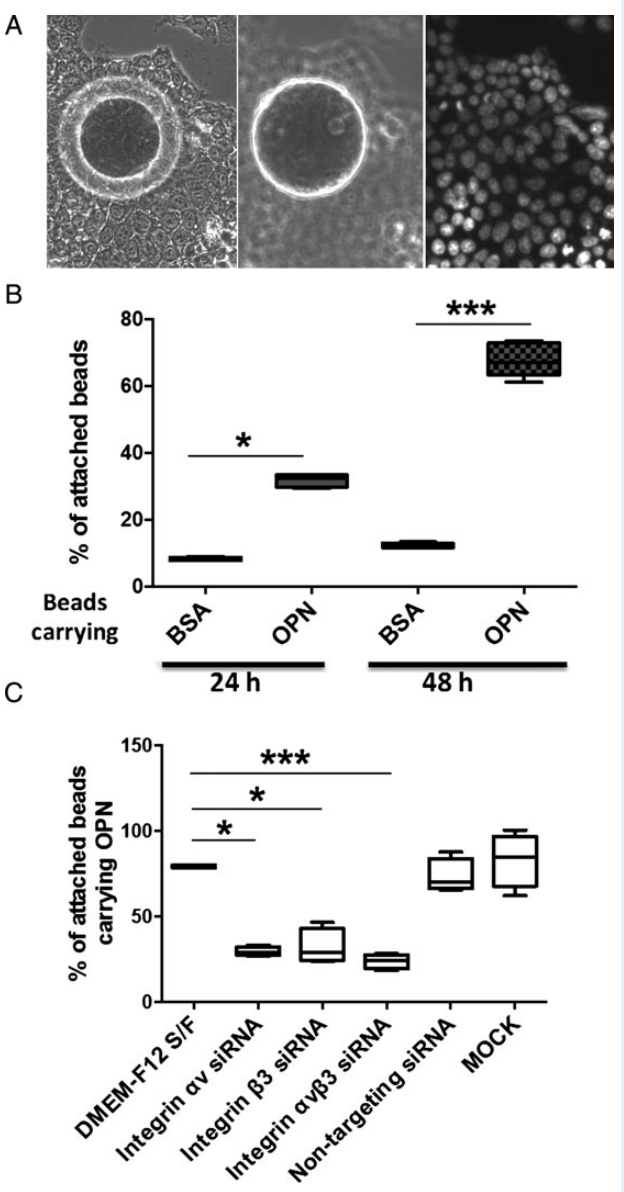

Figure 5 Attachment of OPN-coated beads and the effect of knocking down of integrin αvβ3 in the epithelium. (**A**) A bead is shown attached to the Ishikawa cell monolayer in two planes of focus (phase contrast, left and centre image) with the epithelial monolayer visible beneath. Right, DAPI staining of epithelial nuclei demonstrates no disturbance beneath the bead. (**B**) Attachment rate of beads carrying OPN is significantly higher than control beads carrying BSA ($P = 0.0005$). (**C**) Bead attachment is destabilized by knocking down integrin αvβ3 (either or both subunits, $P = 0.0023$). (B and C) Boxes show quartiles, whiskers show range. *$P < 0.05$, ***$P < 0.001$.

Discussion

Elucidating the molecular interactions that govern the earliest phases of embryo-epithelial cross-talk will be of crucial importance in devising ways to improve implantation rates after embryo transfer (Aplin, 2006; Zhang *et al.*, 2013). An ideal *in vitro* model involves a normal human blastocyst interacting with primary human endometrial cells exhibiting the mid-secretory phase phenotype, both from the same donor (Meseguer *et al.*, 2001). Though we and others (Bentin-Ley *et al.*, 1999) have reported the use of such a model, there are practical difficulties: epithelial cells in primary culture are difficult to propagate and may deviate from the *in vivo* phenotype (Campbell *et al.*, 1988, 2000), while human blastocysts are not available in sufficient numbers to carry out inhibition experiments with suitable controls to elucidate a molecular mechanism.

We (Singh *et al.*, 2010) and others (Dominguez *et al.*, 2010) have made use of a model in which mouse (or rat (Kaneko *et al.*, 2011)) embryos interact with human cells. In both species, embryos implant interstitially after a transient interaction with the epithelium, the endometrium exhibits a maternally controlled receptive phase and aspects of the molecular signatures of the embryo and endometrium are similar (Aplin and Singh, 2008). In this work, we chose to use the well-differentiated Ishikawa cell line in which the cell surface and steroid receptor phenotype has notable similarities to normal epithelial cells (Aplin and Singh, 2008; Singh and Aplin, 2009; Hannan *et al.*, 2010; Singh *et al.*, 2010). We suggest that this could provide a starting point for the identification of molecular pathways that would lead to further investigations in more refined (though less convenient) models, to test the accuracy of the predictions. Nonetheless, mixing species is controversial. In this study, we set out to compare the role of the integrin αvβ3–OPN interaction using three *in vitro* models with the epithelial cell type in common and different interactors: the human embryo, the mouse embryo and an OPN-coated bead proposed to mimic an embryo expressing OPN at the trophectoderm surface. Similar beads have been used *in vivo* in mice to deliver growth

factor signals to the luminal epithelium in the peri-implantation period (Paria *et al.*, 2001). Substantial evidence has demonstrated signalling between attaching embryos and maternal cells to influence gene expression in the latter both *in vivo* and *in vitro* (Meseguer *et al.*, 2001; Wang and Dey, 2006; Singh *et al.*, 2010; Teklenburg *et al.*, 2010).

Using an antibody specific to the heterodimer, we confirmed previous data (Lessey *et al.*, 1992; Aplin *et al.*, 1996b) indicating that αv is present in endometrial epithelium in complex with β3, and showed that its abundance increases at the site of attachment of both human and mouse embryos, suggesting a signalling pathway that is yet to be elucidated. *In vivo*, αvβ3 and OPN immunoreactivity are seen in the apical epithelium in both mouse and human endometrium (Aplin *et al.*, 1996b; Quenby *et al.*, 2007). Furthermore, OPN-binding integrins are present in trophectoderm in blastocyst stage embryos: we have confirmed here that human blastocysts express αvβ3 (Campbell *et al.*, 1995b), and the same is true of mouse, rat and rabbit (Illera *et al.*, 2003; Rout *et al.*, 2004; Kaneko *et al.*, 2011). There is evidence that OPN secreted by uterine epithelial cells binds to mouse embryos (which have been shown to express αvβ3 on trophectoderm (Rout *et al.*, 2004)) and plays a role in the activation of blastocysts for attachment competence (Chaen *et al.*, 2012).

Knock-down experiments were used in all three models to demonstrate a role for αvβ3 and OPN in the attachment of both species of embryo and OPN-coated beads to epithelial cells. The experiments demonstrate that apical attachment stability is impaired when epithelial OPN or αvβ3 levels are reduced, suggesting a model in which OPN acts as a bridging ligand between the uterine epithelium and the trophectoderm. Others have provided evidence that CD98, which binds and activates integrin αvβ3 (Kabir-Salmani *et al.*, 2008), is important for embryo attachment (Dominguez *et al.*, 2010). Our embryo attachment data cannot distinguish a role for secreted maternal OPN in indirectly activating the hatched blastocyst (Chaen *et al.*, 2012), from one in which it acts directly as a bridging ligand between αvβ3 on the maternal surface and αvβ3 or another receptor (such as another integrin or CD44 on trophectoderm (Behzad *et al.*, 1994; Campbell *et al.*, 1995a,b). However, the αvβ3-dependent attachment of OPN-coated beads can most simply be explained by a direct binding interaction. Furthermore, pulldown assays demonstrate this association, though it should be pointed out that only a fraction of the αvβ3 present in lysates made from Ishikawa cells cultured in the absence of embryos is OPN-bound, and so further work will be needed to determine how this might change near attachment sites. In light of the reported fertility of αv-null (Bader *et al.*, 1998) and OPN-null (Liaw *et al.*, 1998) mice, it is significant that embryos can overcome the delay imposed by reducing the abundance of αvβ3 or OPN, becoming stably attached at 48 h.

In all likelihood, implantation relies on a cascade involving several molecular pathways (Aplin, 2000; 2006; Aplin and Kimber, 2004; Aplin and Singh, 2008; Singh and Aplin, 2009). However, timing is critically important; a delay occasioned by late onset or impaired expression of one component of the cascade may lead to loss of embryo viability, or failure to rescue the corpus luteum (Wilcox *et al.*, 1999). In mice the mechanism of corpus luteum rescue is different, and early pregnancy loss is not caused by menstruation; rather, the endometrium becomes refractory (Psychoyos, 1986). We believe an expanded suite of *in vitro* models will play a useful role in the fuller elucidation of contributory mechanisms. The addition of the novel bead attachment protocol has allowed a clear demonstration that integrin αvβ3 is available at the apical epithelial surface for mediating adhesive interactions. Together, the various models will allow further investigation of signalling events leading to modulation of gene expression in maternal epithelial cells at the implantation site, as has been well demonstrated in decidual cells in co-culture with embryos (Teklenburg *et al.*, 2010). Our current work is investigating embryonic signals that modulate the αvβ3–OPN interaction network.

Implantation is a crucial challenge to the embryo; we have previously observed *in vitro* that embryos attach tenuously to Ishikawa cells, but the initial interaction does not always lead to more stable adhesion (Singh *et al.*, 2010). We have proposed that in human, where abnormal embryos are commonplace, this could allow selection by the maternal system of high-quality embryos (Aplin *et al.*, 1996a; Quenby *et al.*, 2002); selectivity in the earliest stage could account for at least a fraction of the many failed embryo transfers in ART.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

Y.J.K. and J.D.A. designed the study. Y.J.K. and K.F. carried out the experimental work. J.C. curated and cultured human embryos. Y.J.K., K.F. and J.D.A. interpreted the results. J.D.A. and Y.J.K. wrote the paper with relevant input from J.C. and K.F.

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Conflict of interest

None declared.

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