Activin A regulates trophoblast cell adhesive properties: implications for implantation failure in women with endometriosis-associated infertility

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BACKGROUND: During implantation, the embryo adheres to the endometrium via cell adhesion molecules (CAMs) present on blastocyst trophoectoderm and endometrial epithelial cells. CAMs, including integrins and extracellular matrix (ECM) ligands, are most likely regulated by hormones, cytokines and growth factors. We hypothesized first that activin A affects the adhesive properties of trophoblast cells and second that alterations in dimeric activin A levels in the uterine cavity could disrupt adhesion, thereby causing implantation failure.

METHODS: This study examined effects of activin A on trophoblast cell adhesion and measured activin A levels in secretory phase uterine washings from women with and without endometriosis (EOS). Activin receptor expression on trophoblast (HTR8) cells was examined by RT–PCR, and adhesive molecules measured by integrin antibody and cell–matrix adhesion assays. Dimeric activin A was measured (enzyme-linked immunosorbent assay) in uterine washings (14 controls and 23 EOS), and βA-subunit localization was verified in endometrial tissues.

RESULTS: Activin receptors are expressed by HTR8 cells. Activin A activated Smad2 in a concentration-dependent manner which was blocked by an activin receptor inhibitor (SB431542). Following activin A treatment (50 ng/ml for 24 h), trophoblast cell surface integrins α1, α2, α3, α5, β1, β2, β4 and αvβ5 were decreased, as was cell binding to the ECM ligands, fibronectin, collagen IV and collagen I (P < 0.05). Activin A was detected in 56.5% of EOS and 21.4% of control washings, with measured levels from 42 to 8481 pg/ml (not significantly different).

CONCLUSIONS: Decreased trophoblast CAM production and adhesion could be caused by dysregulated local activin A levels and may contribute to implantation failure. This could explain, in part, the infertility observed in women with EOS.

Key words: human endometrium / activin A / implantation / integrins / endometriosis

Introduction

Implantation of the embryo into the endometrium is a tightly controlled process requiring synchrony between a receptive endometrium and a healthy mature embryo. Endometrial receptivity occurs for just a brief period of time during the mid-secretory phase of the menstrual cycle: this is the only time the endometrium will accept and support an implanting embryo. During the initial stages of the implantation process, the signals between the blastocyst and the endometrium modulate cell adhesion molecules (CAMs), including integrin receptors and their associated extracellular matrix (ECM) ligands, on both the endometrial and the trophoblastic surfaces. Such molecules are involved in adhesion of the blastocyst to the endometrium (Aplin, 1997; Lessey, 2002; Wang and Armant, 2002).

The precise factors or mechanisms that regulate such trophoblast-endometrial epithelial cell interactions are not fully understood. Although the adhesive process is overall orchestrated by estrogen and progesterone, secreted factors such as cytokines, growth factors and proteases are the likely mediators (Kodaman and Taylor, 2004; Achache and Revel, 2006). Thus, the secretion of such paracrine factors from the endometrial glandular and/or luminal epithelial cells into the uterine cavity during the secretory phase of the menstrual cycle is likely to be essential for implantation success.

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Dimeric activin A, a TGFβ superfamily member, is a homodimer of two βA subunits. Activin A regulates gene transcription via transmembrane activin type 2 receptors (ActRIIA/B) and activin type 1 receptors (ActRIIA or ALK4) and the downstream mediators Smads2 and 3 (Harrison et al., 2005).

In the human endometrium, the βA subunits immunolocalize to the glandular and luminal epithelial cells throughout the menstrual cycle, with staining intensity increasing during the mid-late secretory phase when implantation occurs (Leung et al., 1998; Petraglia et al., 1998; Jones et al., 2000). Furthermore, dimeric activin A protein levels in the uterine cavity increase during the secretory phase (Florio et al., 2003). Activin receptors have been identified on human blastocysts (He et al., 1999), suggesting that activin A secreted into the uterine cavity may act on the blastocyst during implantation.

Endometriosis (EOS) is a gynaecological disorder highly associated with infertility; however, the mechanisms underlying EOS-associated infertility remain unclear. There is increasing evidence to suggest that EOS patients have either an impaired endometrium and/or an abnormal endometrial environment that is functionally unfavourable for implantation and pregnancy progression (Giudice et al., 2002; Kao et al., 2003; Donaghey and Lessey, 2007). Recently, immunoreactive βA subunit was shown to be increased in the glandular epithelium of endometrium from women with EOS (Rombauts et al., 2006). Furthermore, activin A secretion from explanted endometriotic endometrium was increased 7-fold compared with that from normal healthy endometrium, suggesting that activin A secretion from endometrial epithelial cells into the uterine cavity may be increased in EOS patients (Rombauts et al., 2006).

For this study, we hypothesized that activin A plays an important role during the early stages of implantation by affecting the adhesive properties of trophoblast cells, and further, that dimeric activin A levels are increased in the uterine cavity of women with EOS. Taken together, the increased activin A in the uterine cavity of women with EOS could affect trophoblast cell adhesion, and thus implantation. The specific aims of the study were to investigate whether activin A regulates trophoblast adhesion via integrin and ECM ligands and to compare activin A levels in uterine washings from women with and without EOS during the secretory phase.

Materials and Methods

Cell culture

The HTR8/SVneo (HTR8) cell line was derived from human first trimester placenta explant cultures and immortalized using SV40 large T antigen (Graham et al., 1993). The characteristics of this transformed cell line represent a phenotype typical of first trimester trophoblasts, including morphology, cytokeratin expression and responses to TGFβ (Graham et al., 1993). Cells were cultured in RPMI 1640 (Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal calf serum (FCS) and passed at least once following thawing. At ~80% confluency, cells were serum starved for 48 h in serum-free RPMI containing transferrin (10 μg/ml), sodium selenite (25 ng/ml), linoleic acid (4.7 μg/ml) and 0.1% BSA (1 mg/ml) (abbreviated TSL; all from Sigma Diagnostics).

RNA extraction and RT–PCR

Total RNA was extracted from the cultured cells using an RNeasy MiniKit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. All samples were treated with RNase-free DNase (Ambion, Austin, TX, USA) to remove any genomic DNA contamination and were analyzed by spectrophotometry to determine RNA concentration, yield and purity. Total RNA (1 μg) was reverse transcribed at 46 °C for 1.5 h in 20 μl reaction mixture using 100 ng random hexanucleotide primers and 6 IU AMV reverse transcriptase (Roche, Castle Hill, Australia) in the presence of cDNA synthesis buffer (Roche), 1 mmol/l dNTPs (Roche), 10 mmol/l dihydrothioctetol (Roche) and 10 IU ribonuclease inhibitor (RNasin; Promega, Annandale, Australia). The resultant cDNA mixtures were heated at 95 °C for 5 min before storage at -20 °C. Negative controls omitted the reverse transcriptase. Positive control tissues used were: term placenta, secretory phase human endometrium and ovarian granulosa cell tumour tissue (data not shown). Activin primers and conditions have been published previously (Findlay et al., 2001; Fuller et al., 2002).

Gene expression by RT–PCR for all activin receptor subtypes was determined using a conventional PCR block cycler (Hybaid, Middlesex, UK). Each receptor subtype used a 1-μl aliquot of RT product to be amplified in a total volume of 40 μl using 4 μl of RT single-strength PCR buffer (10 mmol/l Tris–HCl, 1.5 mmol/l MgCl2, 50 mmol/l KCl, pH 8.3; Roche), 2.5 mmol/l dNTPs (Gibco, Melbourne, Australia), 0.5 mol/μl sense and antisense primers (Sigma Genosys Australia Pty. Ltd, Castle Hill, Australia) and 2.5 IU Taq-DNA polymerase (Roche). The PCR was performed in three stages as follows: the first stage involved 94 °C for 5 min, x °C for 1 min, where x is the annealing temperature for the individual primer pairs (58–64 °C depending on individual primer) and 72 °C for 2 min; the second stage involved 28–33 cycles of 94 °C for 1 min, 58–64 °C for 1 min and 72 °C for 1 min; and the final stage was 72 °C for 7 min. PCR products including positive controls and DNA ladder were analysed by electrophoresis on a 2% agarose gel (Roche), stained with ethidium bromide and the product size determined to ensure it agreed with previously published data (Fuller et al., 2002; Jones et al., 2002). Product verification was performed using positive control cDNA derived from term placenta, secretory phase human endometrium and granulosa cell tumour tissue and replacement of cDNA with water served as the negative controls (data not shown).

Phosphorylated Smad2/Smad2 western blot

Following cell culture and serum starvation, cells were treated dose-dependently with recombinant human (rh) activin A (carrier-free; R&D Systems Incorporated, MN, USA) diluted in 0.1% BSA/PBS to doses 1, 10, 50 and 100 ng/ml for 24 h. The diluent control included the equivalent amount of 0.1% BSA/PBS for 24 h. The activin inhibitor SB431542 (TOCRIS Bioscience, Northpoint, UK) was used at 10 μM final concentration with or without rh activin A (50 ng/ml) for 24 h. Cells were lysed and homogenized in ice-cold lysis buffer [50 mM Trizma Base (Sigma) pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 0.2% Triton X-100 (Sigma), 0.3% Nonidet P-40 (Sigma) protease inhibitor cocktail set (Calbiochem; EMD Biosciences, NJ, USA)]. Lysates were centrifuged and supernatants were assayed for total protein using the Bradford reagent. Proteins (20 μg per sample) were resolved on a 11% SDS–PAGE gel, transferred to Hybond-P PVDF membranes (GE Healthcare, WI, USA) and blocked in 10% skim milk powder in TBS (1 h, 25 °C). Membranes were incubated overnight at 4 °C with rabbit anti-human phosphoactivated Smad2 (pSmad2; Ser465/467, Cell Signalling Technology, MA USA, #3108; 1:1000 in 5% skim milk/TBS). After washes in TBS and 1% Tween-20 (Bio-Rad) TBS, HRP anti-rabbit secondary antibody (Cell Signalling Technology #3102; 1:2000 in 5% skim milk/TBS) and the ECL Plus Detection System (GE Healthcare) were applied. Membranes were exposed to autoradiography film (Hyperfilm ECL; GE Healthcare) for 5 min. Membranes were then washed in stripping buffer (Re Blot Plus,
Chemicon International, CA, USA) before blocking again in 10% skim milk/TBS, overnight incubation with rabbit anti-human Smad2 (86F7; Cell Signalling Technology, #3122; 1:1000 in 5% skim milk/TBS) and secondary antibody and detection as described above. Films were scanned using Quantity One 1-D Analysis Software (Bio-Rad). Background corrected density OD/mm² for pSmad/Smad2 was obtained and the ratios for treated cells were calculated.

Integrin antibody assays
The Alpha / Beta (α/β) Integrin-Mediated Cell Adhesion Array Combo Kit (ECM532; Chemicon) was used according to the manufacturer’s instructions. This assay measures integrin proteins on the cell surface. We assessed whether activin A regulated trophoblast expression of certain integrin subunits (α1, α2, α3, α4, α5, αv, β1, β2, β3, β4 and β6) and integrin complexes/heterodimers (αvβ3, αvβ5 and α5β1). Briefly, HTR8 cells were cultured and serum starved as described above. Cells were then cultured in RPMI/TSL with rh activin A (R&D Systems Inc.) at a final concentration of 50 ng/ml. A diluent control, without activin A, was included in every assay for comparison. Following the 24 h incubation, cells were washed twice in PBS without calcium or magnesium (Ca²⁺/Mg²⁺; PBS⁻), harvested with a mild 5 mM trypsin–EDTA/PBS solution, spun down at 1500 rpm for 5 min, counted and diluted to a final concentration of 5 x 10⁵ cells/ml RPMI/10% charcoal stripped (cs) FCS. From this cell suspension, each well containing mouse anti-alpha (α) or anti-beta (β) integrin received 100 μl containing 5 x 10⁴ cells, as did the BSA-coated negative control wells. The plate was then incubated for 1.5 h at 37°C in a CO₂ incubator, after which it was gently washed with assay buffer. Cells were then stained with a Cell Stain Solution (provided in kit), incubated for 5 min, and then the solution was removed, the cells washed with dH₂O, and air dried. Extraction buffer (100 μl) was added to each well and left to shake for 5–10 min. The absorbance was determined at 540–570 nm on a plate reader. Each culture experiment (n = 3) included triplicate wells for each treatment and was assayed in n = 3 integrin assays for each of the integrin subunits or heterodimers.

Cell–matrix adhesion assays
Regulation of trophoblast adhesion was assessed following activin A treatment as above using CytoMatrix Screening Kit, ECM105 adhesion assays (Chemicon). The HTR8 trophoblast cells were cultured, treated, counted and diluted to 5 x 10⁵ cells/ml RPMI/10% csFCS. The diluted cell suspension (100 μl) was added to each well of plates coated with various matrix substrates [fibronectin (FN), laminin (LN), vitronectin (VN), collagen I (COLI) and collagen IV (COLIV)]. Following incubation for 1.5 h at 37°C in a CO₂ incubator, the wells were washed three times with PBS containing Ca²⁺/Mg²⁺ and then 0.2% crystal violet in 5% ethanol (100 μl) was added to each well and incubated for 5 min at room temperature. Stain was then removed from the wells, and the cells again washed three times with PBS. The bound cells (stained) were solubilized by an addition of 100 μl solubilization buffer (a 50/50 mix of 0.1 M NaH₂PO₄, pH 4.5, and 50% ethanol) to each well and incubated at room temperature for 5 min. Adhesion was determined based on the absorbance of the stain measured at 560 nm. BSA-coated wells were included for each treatment in the assays as negative controls. Each experiment included triplicate wells per treatment and the entire culture experiments (n = 4) for FN, COLI and COLIV were carried out in n = 4 adhesion assays.

Endometrial washings and tissue collection
Control endometrial (uterine) washings were collected from normal cycling women during the mid-secretory phase of the menstrual cycle undergoing benign gynaecological procedures, mostly laparoscopic sterilizations, who were of known fertility (n = 14, mean age 33 ± 6). There was no evidence of EOS or other endometrial pathologies during surgery, which was confirmed by pathology. Uterine washings were also collected from women with various stages of EOS (n = 23), ranging from EOS stage I to IV, as confirmed by the surgeon at laparoscopic surgery and subsequent pathological reports. Of these women, all presented as infertile except three women who were presenting for investigation of pain, and therefore their fertility status was unknown. The mean age of all women with EOS was 33 ± 6 years, and all washings were collected during the secretory phase of the menstrual cycle, confirmed by pathology. The uterine washing procedure involves inserting a fine catheter through the cervix into the uterine cavity during the mid-secretory phase of the menstrual cycle, and then washing the cavity with 5 ml sterile saline solution (0.9%), which is retrieved. From each woman, a small sample of endometrial tissue was collected in formalin for histological and immunohistochemical analysis. Endometrial tissue was dated from the women’s menstrual history and confirmed histologically by examination based on the Noyes criteria (Noyes et al., 1975). Women who participated in the study did not undergo any additional procedure to that of routine investigatory methods, and ethical approval was obtained from both Southern Health and Epworth Hospital Ethics Committees. All women gave informed consent for the study.

Uterine washing sample preparation
Uterine washings were stored on ice until centrifugation (900 g for 5 min) to remove debris. The supernatant was collected and 0.1% protease inhibitor cocktail set (Calbiochem) added. Aliquots were then snap frozen and stored at −80°C until use. Samples were measured for total protein content using a standard Bradford assay (Sigma). A 500-μl aliquot of each sample was then concentrated 3.5-fold using Microcon® Centrifugal Filter Units (3 kDa cutoff; Millipore, MA, USA), which require centrifugation at 14 000g for 100 min.

Activin A enzyme-linked immunosorbent assay
Activin A was measured in uterine washings using a specific enzyme-linked immunosorbent assay (ELISA), which detects dimeric activin A (Knight et al., 1996), according to the manufacturer’s instructions (Oxford Bio-Innovations, Oxfordshire, UK) with some modifications as described previously (O’Connor et al., 1999). Additional modifications were made to measure activin in human endometrial washing samples. The standard was diluted in 0.9% saline to match the samples that were measured in duplicate. Serial dilutions of a pool of human endometrial washings resulted in a linear dose–response curve which ran in parallel to the standard. Test samples of washings spiked with rh activin A resulted in average recoveries of ~95% (data not shown). The average intra-assay % coefficient of variation (CV) was 6.8%, the inter-plate % CV was 9.2% (n = 3 plates) and the limit of detection was 10 pg/ml.

Statistical analyses
Data are expressed as the mean ± SEM fold change for each treatment compared with control. For each independent experimental treatment, values were expressed as a percentage of controls, and statistical analysis was then carried out on the data from different experiments. Values were assessed for normal distribution, and if so, an unpaired Student’s t-test was performed comparing control versus activin A-treated cells. If values were not normally distributed, the Mann–Whitney non-parametric test was performed. For both tests, P < 0.05 was taken as significant.
Results

Activin receptors are expressed by HTR8 trophoblast cells

To ensure HTR8 trophoblast cells express the appropriate activin receptors (ActRIIA, ActRIIB, ActRIA and ALK4), semi-quantitative RT–PCR was performed (Fig. 1). Messenger RNA for each of the activin receptors was expressed by HTR8 trophoblast cells at the previously published size (Fuller et al., 2002; Jones et al., 2002).

Activin A treatment causes pSmad2 in HTR8 cells

HTR8 cells do not secrete detectable levels of activin A, as determined by activin A ELISA (data not shown). rh activin A was added to HTR8 cells at doses 1, 10, 50 and 100 ng/ml for 24 h, and pSmad2 was detected by western blot analysis (Fig. 2). Densitometry showed that there was no overall effect of activin A on total levels of Smad2. Addition of activin A (10–100 ng/ml) to cells stimulated the phosphorylation of Smad2 (Fig. 2). A dose of 50 ng/ml was chosen as an appropriate dose for subsequent experiments. Addition of SB431542, an activin inhibitor, reduced pSmad2 in response to 50 ng/ml activin A (Fig. 2A and B).

Activin A decreases integrin protein subunits on HTR8 trophoblast cells

Integrins are involved in the implantation process. To elucidate whether activin A may be involved in the regulation of these monomeric or heterodimeric receptors at the protein level, specific α and β integrin binding assays were used to evaluate the effect of activin A on integrin production in HTR8 cells. Initial experiments using untreated HTR8 cells identified that these cells bound most efficiently to integrin subunits/heterodimers α1, α2, α3, α5, αv, β1, β2, β4, αvβ3, αvβ5 and α5β1 and not to α4, β3 or β6. These latter three subunits were not further included in the study (data not shown). Following activin A treatment for 24 h, most integrin subunits tested showed a significant down-regulation of cell surface production (Fig. 3A and B). Specifically these were α subunits α1, α2, α3 and α5 (all P < 0.01; Fig. 3A) and β subunits β1 (P < 0.01), β2 (P < 0.05), β4 (P < 0.001) and αvβ5 (P < 0.05) (Fig. 3B). Cell surface production of integrin subunit αv and heterodimers αvβ3 and α5β1 were not significantly decreased with activin A treatment when compared with control, untreated cells.

Activin A decreases trophoblast cell binding to FN, COLIV and COLI

Cell adhesion to the ECM components, FN, LN, VN, COLI and COLIV, was assessed using matrix-binding assays to determine whether activin A regulates trophoblast adhesion to these ligands. Initial experiments identified that untreated HTR8 cells under control conditions bound most efficiently to FN and the COL (I and IV) compared with LN and VN (Fig. 4A): therefore, LN and VN were not assessed further. There was a significant decrease in binding to FN (P < 0.01; Fig. 4B), COLIV (P < 0.01; Fig. 4C) and COLI (P < 0.05; Fig. 4D) following HTR8 treatment with activin A for 24 h (Fig. 4). When SB431542 (an activin inhibitor; 10 μM) was added together with activin A, the binding to FN, COLIV and COLI was unchanged when compared with the adhesion observed using untreated control cells (Fig. 4B–D).

Activin A in the uterine cavity in a cohort of women with EOS

Secreted dimeric activin A was measured by ELISA in uterine washings from women with and without EOS. This particular activin A ELISA was chosen as it provided the highest sensitivity (>10 pg/ml), which was important given the low levels of activin A present in uterine fluid. Activin A in the uterine fluid of 14 control women and

**Figure 1** Activin receptors are expressed by HTR8 trophoblast cells. HTR8 trophoblast cells grown under serum-free conditions for 24 h express mRNA for all activin receptors (ActR1A, ALK4 and ActRIIA/B), as determined by semi-quantitative RT–PCR. The product analysis for each gene showed a single band at the expected band size.

**Figure 2** pSmad2 of HTR8 trophoblast cells. (A) Treatment of HTR8 trophoblast cells with activin A (ActA; 1, 10, 50 and 100 ng/ml) for 24 h results in a dose-dependent increase in pSmad2 as shown by western blot. Addition of activin inhibitor, SB431542 (SB: 10 μM) in conjunction with ActA (50 ng/ml), decreased pSmad2 compared with ActA alone. The lower band represents total Smad2 which is not changed with ActA treatment. (B) Densitometric analysis of representative western blot in (A). Each bar represents the mean of two experiments assayed in duplicate.
Activin A and trophoblast adhesion

Discussion

During implantation, successful blastocyst adherence to a receptive endometrium requires CAMs on the trophoblast surface. Integrins are considered to be primary mediators of adhesion on the apical surface of trophoblast cells (Wang and Arment, 2002); however, the regulation of these molecules remains largely unknown. Here, we report the novel finding that activin A regulates the adhesive properties of trophoblast cells, causing a significant decrease in the production of cell surface integrin subunits (α1, α2, α3, α5, β1, β2 and β4) and in the binding of cells to ECM ligands (FN, COLIV and COLI). Furthermore, this study has demonstrated that activin A levels may be dysregulated in the uterine cavity of a cohort of women with EOS, a disease highly associated with infertility. These findings could explain, in part, the implantation failure in women with high levels of activin A present in their uterine cavity during the peri-implantation period.

For ethical reasons, studying and understanding the initial events of the implantation process in humans is difficult, particularly as there are no trophoblast cell lines available. In the current study, the HTR8 trophoblast cell line, a transformed cell line derived from first trimester placenta culture explants (Graham et al., 1993), was chosen to examine the effect of activin A on the adhesive properties potentially involved in human implantation. This choice was based on two factors; first, HTR8 cells do not express HLAG, a marker of an extravillous trophoblast cells that have invaded into the decidua, unless they are grown on an ECM such as Matrigel® (Graham et al., 1993). We were interested in modelling the adhesion of blastocyst trophoblast cells to the endometrial epithelium, and since there are no such cell lines available and since there is no way of culturing human trophoblast cells, we chose a human trophoblast cell line to represent the preimplantation trophoblast cells. Second, these cells were shown to express mRNA for all activin receptors, as do human blastocysts (He et al., 1999). Activin A signalling is activated via its binding to its cell surface receptor. Activin A induced the phosphorylation of Smad2 in the HTR8 cells, suggesting that all of activin A signalling components were present in these cells. In addition, the HTR8 trophoblast cells do not secrete detectable levels of activin A, suggesting any effect/responses noted would be due to the addition of activin A and not to endogenous activin A.

Various integrin subunits and heterodimers have been identified on the blastocyst surface and are considered essential for the adhesion between the trophodermal cells of the blastocyst and the endometrial epithelium (see review: Lessey and Castelbaum, 2002). Integrin subunits β1, β5, α3 and possibly αv are expressed by human preimplantation blastocysts, suggesting heterodimers α3β1, αvβ1 and/or αvβ5 could be involved in blastocyst adhesion (Campbell et al., 1995; Kimber and Spanswick, 2000). The expression and regulation of integrins is dependent on secreted factors within the cell microenvironment, although precisely which factors are involved in integrin regulation remains largely unknown. In the present study, trophoblast cell surface production of integrins β1, β5, α3 and αvβ5 were decreased by the action of activin A, providing evidence suggesting that activin A is involved in the regulation of these particular integrin subunits on the trophoblast cell surface. However, we observed no significant decrease in trophoblast cell surface production of integrin subunit αv or heterodimers αvβ3 and α5β1 following treatment with activin A, implying that activin A does not regulate these integrins on HTR8 cells. Integrin heterodimers can bind more than one ligand and individual ligands are often recognized by more than one integrin suggesting shared functional properties (Reddy and Mangale, 2003). It is possible that in HTR8 cells, integrin β1 dimerizes with an integrin other than α5 and one that is not regulated by activin A, and this may be the reason why no significant effect was seen in the regulation of integrin α5β1.

In addition to cell–cell adhesion, some integrins, particularly β1 subunits, are involved in cell–ECM adhesion, a process known to be involved in the anchorage between the blastocyst and the endome-trium. Integrins recognize many different ECM ligands and interact with...
these through their three-amino-acid sequence, Arg-Gly-Asp (RDG) (Ruoslahti and Piersbacher, 1987). Through this interaction, trophoblast cells are capable of adhering to the several ECM components that are up-regulated in peri-implantation endometrium, including FN, LN and COLIV (Rider et al., 1992; Fazleabas et al., 1997) to strengthen adhesive activity. In the current study, control untreated HTR8 trophoblast cells bound preferentially to FN, COLIV and COLI, making these cells an appropriate choice to assess the effect of activin A on binding to these ECM ligands, with the exception of LN. Importantly when these trophoblast cells were treated with activin A, there was a decrease in cell binding or adhesion to all three ECM ligands—FN, COL IV and COL I—and the binding to each remained unchanged when an activin inhibitor was included. This implied that the result was specific to activin A and that cell surface adhesion/integrin molecules were changed. With a reduction in trophoblast binding to these ECM ligands following activin A treatment, it is possible that activin A affects the adhesive link between trophoblast cells and the peri-implantation endometrium, resulting in implantation failure.

EOS is a gynaecological disorder characterized by the presence of ectopic endometrial tissue at various sites within the peritoneal cavity, and which is highly associated with infertility. In the eutopic endometrium of women with EOS, the activin A subunit is increased in epithelial cells compared with that observed in women without EOS (Rombauts et al., 2006). In addition, activin A secretion from explanted endometrium and endometrial cells is higher from women with EOS compared with secretion levels from explants and cells from control women (Rombauts et al., 2006). In the present study, dimeric activin A was measured by ELISA in uterine washings from women with and without EOS and, interestingly, was detectable in 56% of the EOS washings compared with only 21.4% of the control washings (not significantly different). In particular, very high levels were detected in three of the women with EOS, suggesting that there may be a cohort of women with EOS-associated infertility in

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**Figure 4** Effect of activin A on adhesion of HTR8 trophoblast cells to FN, COLIV and COLI. (A) Adhesion profile of HTR8 trophoblast cells to FN, COLIV, COLI, VN and LN under unstimulated conditions. Data are representative of three separate experiments assayed in triplicate. HTR8 cells were cultured with/without activin A (ActA; 50 ng/ml) or ActA + SB431542 (SB; 10 μM) for 24 h and their adhesion to FN (B), COL IV (C) and COL I (D) screened. Data for each are represented as the mean percentage of control (100%) ± SEM for combined data from four independent experiments. Each sample was assayed in triplicate. *P < 0.05, **P < 0.01.

**Figure 5** Activin A levels in uterine washings from EOS and control women. Scatter plot showing the levels of detectable dimeric activin A (pg/ml) in uterine washings from controls and EOS. Bars represent the mean levels for each group. The level of sensitivity of the assay is marked by the dotted line at 10 pg/ml.
whom activin A secretion is disturbed. Likewise, both endometrial IL-11 and LIF are dysregulated in some but not all women with EOS (Dimitriadis et al., 2006). However, in a separate study, LIF and IL-11 levels in uterine washings from women with and without EOS were not significantly different between the groups (Mikolajczyk et al., 2006).

The levels of activin A in the uterine washings did vary considerably, particularly among the EOS group. A likely explanation for this is the very different aetiologies of this disease in different women. This study did not take into account symptoms or location of EOS, which are both factors that might affect the activin A levels. Other limitations of our study include the relatively small sample size, which is attributed to the difficulty in the collection of appropriate washings. Future studies with larger, well-defined cohorts of EOS patients may provide further elucidation of the importance of activin A and other biomarkers in uterine washings of EOS patients.

In conclusion, this study has shown that in the presence of activin A, trophoblast cells show a decrease in the production of certain integrins and ECM ligands, suggesting that high levels of activin A may be disruptive to adhesion, and thus contribute to implantation failure. This finding may explain why some women with EOS, who have high levels of activin A in the uterine cavity, are susceptible to implantation failure and infertility.

Authors’ roles
C.J.S.: performed experiments, data analysis and drafted manuscript; L.A.S.: assisted with drafting manuscript and assisted in concept design; N.J.H.: assistance with collection of uterine washings; A.E.O.: performed experiments and data analysis; L.R.: collection of uterine washings; E.D.: concept design, oversaw experiments and data analysis and assisted in drafting manuscript.

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