Uterine focal adhesions are retained at implantation after rat ovarian hyperstimulation

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Abstract

Controlled ovarian hyperstimulation is an essential component of IVF techniques to ensure proliferation and development of multiple ovarian follicles, but the effects of these hormones on the endometrium are largely unknown. During normal pregnancy in rats, there are significant changes in the basal plasma membrane of uterine epithelial cells (UECs) at the time of receptivity, including loss of focal adhesions. This enables the UECs to be removed from the implantation chamber surrounding the blastocyst, thus allowing invasion into the underlying stroma. This study investigated the influence of ovarian hyperstimulation (OH) on the basal plasma membrane of UECs during early pregnancy in the rat. Immunofluorescence results demonstrate the presence of paxillin, talin, integrin $\beta 1$ and phosphorylated FAK (Y397FAK) in the basal portion of UECs at the time of implantation in OH pregnancy. TEM analysis demonstrated a flattened basal lamina and the presence of focal adhesions on the basal surface at this time in OH pregnancy. Significantly low full-length paxillin, high paxillin δ and integrin $\beta 1$ were seen at the time of implantation in OH compared with those in normal pregnancy. The increase in paxillin δ suggests that these cells are less mobile, whereas the increase in integrin $\beta 1$ and Y397FAK suggests the retention of a stable FA complex. Taken together with the increase in morphological focal adhesions, this represents a cell type that is stable and less easily removed for blastocyst implantation. This may be one mechanism explaining lower implantation rates after fresh embryo transfers compared with frozen cycles.

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Introduction

An essential part of the ability of uterine epithelial cells (UECs) to become receptive to an implanting blastocyst is the changes in all membrane domains of the cell, including the basal plasma membrane (Shion & Murphy 1995). In species that have displacement penetration such as rodents and humans (Schlafke & Enders 1975), the UECs must be removed for the implanting blastocyst to invade the underlying stroma. This removal was thought to be mediated via apoptotic mechanisms (Parr *et al.* 1987); however, recent evidence suggests that luminal UECs surrounding the implanting blastocyst undergo entosis (Li *et al.* 2015). Regardless of the mode of removal of UECs, it is necessary for the UECs to become less adherent to the underlying basal lamina, and then the UECs in the region immediately surrounding the implantation chamber are removed. Previous studies in rat UECs during early pregnancy and in response to ovarian hormones showed that at the time of implantation and in response to progesterone, the basal lamina thickens, becomes highly tortuous and loses focal adhesions (Shion & Murphy 1995, Kaneko *et al.* 2008).

Focal adhesions (FAs) are key structural elements that play a role in cell-matrix adhesion. These specialised structures consist of several proteins that connect the actin cytoskeleton to the extracellular matrix (ECM). Intracellular proteins associated with FAs include actopaxin, vinculin, paxillin and talin (Turner 2000) and membrane-bound integrins, which span the plasma membrane and make contact with the ECM of the basal lamina (Vuori 1998, Brown & Turner 2004) and all of which have complex interactions (Zaidel-Bar *et al.* 2007, Kuo *et al.* 2011).

Paxillin is a linker protein that connects to integrins via focal adhesion kinase (FAK) and actin stress fibres via actopaxin and vinculin (Turner *et al.* 1990, Turner 2000, Schaller 2001). There are four different isoforms of paxillin with paxillin α being the most widely expressed (Mazaki *et al.* 1997, Schaller 2001, Brown & Turner 2004). Paxillin δ is an internal translation product of paxillin α that is found only in epithelial cells (Brown & Turner 2004, Tumbarello *et al.* 2005), and when overexpressed, leads to the inhibition of cell migration (Sorenson & Sheibani 1999, Tumbarello *et al.* 2005). Talin is another intracellular protein associated with FAs and provides a direct link between the cytoplasmic domain of integrin

and intracellular actin fibres (Nayal *et al.* 2004). Talin is also involved in integrin activation (Tadokoro *et al.* 2003), which is essential in the establishment of FAs (Yamada & Geiger 1997, Critchley 2009).

Integrins are large membrane-spanning proteins composed of α and β subunits that mediate cell–cell and cell–extracellular matrix adhesion. *In vitro* studies have shown that integrin $\beta 1$ and $\beta 3$ colocalise and interact with talin at FAs (Calderwood *et al.* 1999, Critchley 2000, Calderwood 2004). Previous studies in humans, rodents and other mammals have established that integrin $\alpha_v\beta_3$ is highly expressed at the time of implantation and is a well-known marker of uterine receptivity (Bowen *et al.* 1996, Fazleabas *et al.* 1997, Lessey 1997, Srinivasan *et al.* 2009). Integrin $\beta 1$ has previously been localised to the basal plasma membrane of rat UECs on day 1 of pregnancy and in response to oestrogen stimulation (Kaneko *et al.* 2011).

Focal adhesion kinase (FAK) is a cytosolic protein tyrosine kinase associated with the FA complex and is involved in many aspects of cell migration, invasion and signalling (Cary & Guan 1999, Hauck *et al.* 2002). Previous work in rat UECs during early pregnancy and in response to ovarian hormones has shown that FAK is found apically at the time of implantation and in response to progesterone. This apical localisation of FAK is suggested to be involved in integrin–integrin binding between UECs and blastocyst, which is an essential part of implantation (Kaneko *et al.* 2012). Human studies have also shown an increase in FAK staining within the luminal and glandular UECs in the mid-secretory phase of the menstrual cycle indicating a role in human uterine remodelling and implantation (Orazizadeh *et al.* 2009).

For FAK to control intracellular signalling pathways, autophosphorylation must occur, and this can happen on a number of residues including tyrosine-397. This autophosphorylation then activates many of the kinase-dependant functions of FAK (Katz *et al.* 2003, Brami-Cherrier *et al.* 2014). Specifically, phosphorylation of FAK at Y397 (Y397FAK) leads to the activation of integrins in a variety of cell types *in vitro* and *in vivo* (Chan *et al.* 1994, Schaller *et al.* 1994, Eide *et al.* 1995, Schlaepfer *et al.* 1999). Although several studies have investigated FAK in the endometrium (Orazizadeh *et al.* 2009, Kaneko *et al.* 2012), currently no studies have examined the physiologically active form of FAK, Y397FAK, in the endometrium. However, in the myometrium, an increase in phosphorylation of FAK is associated with stretch-induced activation of human myometrial cells (Li *et al.* 2009). In human placental cytotrophoblast cells, it was found that Y397FAK, rather than overall FAK levels, is associated with invasion ability and that under conditions of hypoxia *in vitro* and pre-eclampsia *in vivo*, there was a reduction in Y397FAK (Ilić *et al.* 2001).

During IVF procedures, controlled ovarian hyperstimulation (COH) is required to stimulate oocyte production and maturation. COH has a detrimental

effect on the uterine lining, which impedes blastocyst implantation. This is confirmed in several studies that show a significant decrease in implantation rate when embryos are transferred during the COH cycle, compared with those transferred in subsequent cycles (Paulson *et al.* 1990, Check *et al.* 1994, 2010, Shapiro *et al.* 2011, 2014). However, the mechanisms underlying the effect of COH on uterine receptivity are currently unknown.

Figure 1 Immunofluorescence micrographs and western blotting analysis of paxillin on days 1 and 3 of normal and OH pregnancy. Paxillin staining (green) is seen as a basal band below uterine epithelial cells (e) on day 1 of normal pregnancy (D1, arrowhead). Similar basal staining is also seen below UECs (e) on day 1 after OH (OH1, arrowhead). Protein analysis from isolated UECs showed 68 kDa (full-length paxillin) and 46 kDa (paxillin δ) bands. When normalised to β -actin, there is no difference in either 68 kDa or 46 kDa isoforms of paxillin between normal and OH pregnancy on day 1. On day 3 of normal (D3) and OH pregnancy (OH3), a small amount of cytoplasmic paxillin staining is seen in UECs (e). Western blotting analysis demonstrates full-length paxillin (68 kDa) and a small amount of paxillin δ (46 kDa). However, there is no difference in amount of either protein in isolated UECs of normal compared with OH pregnancy. Green staining, paxillin; blue, nuclei; scale bar = 20 μ m.

Figure 2 Immunofluorescence micrographs and western blotting analysis of paxillin on days 6 and 7 of normal and OH pregnancy. Paxillin staining (green) is seen as a prominent basal band below UECs (e) on day 6 of OH pregnancy (OH6, arrowhead). There is no immunofluorescent staining on day 6 of pregnancy (D6). Western blotting analysis of isolated UECs from day 6 of normal and OH pregnancy showed full-length protein (68 kDa) and paxillin δ isoform (46 kDa) bands. When normalised to β -actin, there was a significant reduction in full-length paxillin and an increase in paxillin δ in OH compared with normal pregnancy on day 6. On day 7 of normal (D7) and OH pregnancy (OH7), paxillin is seen as a basal band below the UECs (e, arrowhead). Protein analysis from isolated UECs showed 68 kDa and 46 kDa bands on day 7 of OH pregnancy and only the full-length protein from day 7 of pregnancy. When normalised to β -actin, there was no difference in 68 kDa paxillin protein, but a significant increase in paxillin δ on day 7 in OH compared with normal pregnancy. Green staining, paxillin; blue, nuclei; scale bar = 20 μ m; * P < 0.05; ** P < 0.001.

This study used a rat ovarian hyperstimulation (OH) model to investigate the effects of this protocol on ultrastructural FAs and related proteins in UECs at the time of receptivity. Previous studies using this OH model have shown a lack of implantation sites in treated animals, suggesting that this treatment interferes with normal uterine receptivity (Jovanovic & Kramer 2010). Significant changes in uterine fluid and

ion transport (Lindsay & Murphy 2014) and change in important uterine adhesion molecules (Sendag *et al.* 2010, Biyiksiz *et al.* 2011) are also observed in OH pregnancy compared with normal pregnancy. There are, however, no data investigating ultrastructural or molecular changes of the basal plasma membrane of UECs at the time of implantation after OH compared with normal pregnancy.

Materials and methods

Animals

Adult female virgin Wistar rats were housed in a controlled environment with a 12-h light/darkness cycle and given food and water *ad libitum*. In one group of rats, vaginal smears were used to determine which animals were in pro-oestrus and these rats were mated overnight with a male of proven fertility. Mating was confirmed the following morning by the presence of sperm in a vaginal smear, this was designated as day 1 of pregnancy (D1).

A second group of animals were smeared daily until at least 2 continuous 4-day oestrous cycles were noted. At noon on the day of mid-dioestrus, an intraperitoneal injection of 20 IU of serum gonadotropin (PMSG; Folligon; Intervet Australia, Vic, Australia) was delivered, followed 24 h later by an intraperitoneal injection of 20 IU of human chorionic gonadotropin (hCG; Chorulon; Intervet, Australia). These ovarian hyperstimulated female rats were then mated overnight and the presence of sperm in the vaginal smear the following morning confirmed pregnancy, this was designated as day 1 of ovarian hyperstimulated pregnancy (OHD1).

Five animals per group (normal pregnancy D1, 3, 6 and 7; OH pregnancy OHD1, 3, 6 and 7) were killed with an intraperitoneal injection of sodium pentobarbitone (Troy Laboratories, Australia) and uterine tissue removed and processed as described below. All animal procedures were approved by the University of Sydney Animal Ethics Committee.

Immunofluorescence microscopy

Uterine horns randomly selected for light microscopy were immediately placed in cold 0.1 M phosphate buffer (pH 7.4) and cut into 5 mm pieces. Uterine pieces were covered with OCT (Tissue Tek, USA) and frozen in supercooled isopentane before being stored in liquid nitrogen until use. Sections, 8 μ m thick, were cut from at least 2 blocks per animal and placed on gelatin-chrome alum-coated slides. Sections were randomly allocated to each antibody or control IgGs. After fixing in 4% paraformaldehyde solution for 10 min at room temperature (RT) for paxillin, talin or Y397FAK, slides were washed with phosphate-buffered saline (PBS). Non-specific binding was blocked with PBS/1% bovine serum albumin (BSA) for 30 min at RT. Primary antibodies were applied at a concentration of 14 μ g/mL (Paxillin, Sigma-Aldrich), 16 μ g/mL (Talin, Sigma-Aldrich), 20 μ g/mL (Focal Adhesion Kinase, phosphor Y397, Abcam), 20 μ g/mL (normal rabbit IgG, Sigma-Aldrich) or 16 μ g/mL (normal mouse IgG, Sigma-Aldrich) overnight at

Figure 3 Immunofluorescence micrographs and Western blotting analysis of talin on days 1 and 3 of normal and OH pregnancy. Basal talin staining is seen in uterine epithelial cells (e) on day 1 of normal pregnancy (D1), day 1 of OH pregnancy (OH1) and day 3 of OH pregnancy. Cytoplasmic talin staining is seen in UECs on day 3 of normal pregnancy (D3). Protein analysis from isolated UECs showed talin as 225 kDa (full length) and 190 kDa (proteolytically cleaved product) with no difference seen between OH and normal pregnancy on either days 1 or 3. Green staining, talin; blue, nuclei; scale bar = 20 μ m.

4°C. Sections were then washed in PBS and incubated in fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse IgG secondary antibodies for paxillin and talin (1 μ g/mL, Jackson ImmunoResearch Laboratories) or FITC-conjugated goat-anti-rabbit IgG secondary antibodies for Y397FAK (3 μ g/mL, Jackson ImmunoResearch Laboratories) for 1 h at RT in the darkness to prevent quenching.

Sections randomly allocated for colocalisation experiments between integrin β 1 and talin were fixed in PFA for 10 min, washed in PBS and blocked in PBS/1% BSA for 30 min at RT before being incubated in integrin β 1 antibodies (1 μ g/mL, Abcam) overnight at 4°C. After washing in PBS, sections were incubated in FITC-conjugated goat-anti-rabbit IgG secondary antibodies for 2 h at RT, washed in PBS and incubated in 16 μ g/mL talin antibodies overnight at 4°C.

All sections were washed in PBS, mounted with VECTASHIELD-containing DAPI (Vector, Burlingame, CA,

USA) coverslipped and observed immediately using the Zeiss Deconvolution Microscope (Carl Zeiss). Images were acquired using a Zeiss AxioCam HRM digital camera and Zen software (Carl Zeiss), and micrographs were produced using Photoshop software (Adobe Systems).

Western blotting analysis

Uterine horns randomly allocated for protein analysis were opened longitudinally to expose the luminal epithelium and gently washed with phosphate buffer. The epithelium was scraped using a scalpel blade and placed into 100 μ L of mammalian cell lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 150 mM sodium chloride, 0.1% sodium dodecyl sulphate (SDS), 0.5% deoxycholic acid sodium salt solution and 1% IGEPAL; Sigma)-containing 10% phosphatase inhibitor cocktail (PhosSTOP, Roche, USA) and 1% protease inhibitor cocktail (Sigma). The cell lysis buffer solution was stored on

Figure 4 Immunofluorescence micrographs and Western blotting analysis of talin on days 6 and 7 of normal and OH pregnancy. Basal talin staining is seen in uterine epithelial cells (e) on days 6 and 7 of normal and OH pregnancy. Protein analysis from isolated UECs showed talin as 225 kDa (full-length) and 190 kDa (proteolytically cleaved product) with no difference seen between OH and normal pregnancy on either days 6 or 7. Green staining, talin; blue, nuclei; scale bar = 20 μ m.

Figure 5 Immunofluorescence micrographs showing the location of talin (green), integrin $\beta 1$ (red) and the co-localisation (yellow) between these proteins on days 1 and 6 of normal pregnancy and after OH. There is basal integrin $\beta 1$ staining of the luminal epithelial cells on day 1 of normal and OH pregnancy as well as on day 6 of OH pregnancy. There is no integrin $\beta 1$ staining of the luminal epithelial cells on day 6 of normal pregnancy. Talin and integrin $\beta 1$ co-localise (arrowhead) on days 1 and 6 of normal pregnancy and on day 6 after OH. Green staining, talin; red, integrin $\beta 1$; blue, nuclei; scale bar = 20 μ m.

ice for 10 min before being centrifuged to remove cellular debris, aliquoted and stored at -80°C until use. Randomly selected regions of the remaining uterine horn were cut into 5 mm pieces, frozen and stored in liquid nitrogen as stated previously. Blocks were cut, stained with routine haematoxylin and eosin and observed to validate the removal of luminal epithelial cells only.

Protein concentration was determined using a Micro BCA Protein Assay Kit (Pierce Biotechnology, USA), and 20 μ g was loaded onto a 4–20% pre-cast gel (Bio-Rad) and electrophoresis was carried out according to the method of Laemmli (Laemmli 1970). Protein was transferred electrophoretically to polyvinylidene-fluoride microporous membranes (Millipore). Membranes were blocked with Tris-buffered saline (TBS)-containing 0.05% Tween20 (TBS-T)/5% skim milk for 1 h at RT. Mouse-anti-paxillin (7 μ g/mL), mouse-anti-talin (1.1 μ g/mL), rabbit-anti-integrin $\beta 1$ (0.4 μ g/mL) and rabbit-anti-Y397FAK (1 μ g/mL) antibodies were diluted with TBS-T/1% skim milk and membranes were incubated overnight at 4°C . Membranes were washed in TBS-T and then incubated in 0.2 μ g/mL HRP-conjugated goat anti-mouse IgG (GE Healthcare) or 0.25 μ g/mL horseradish peroxidase (HRP)-conjugated goat-anti-rabbit IgG (Dako) diluted in TBS-T/1% skim milk for 2 h at RT. After the final wash in TBS-T, membranes were incubated in Immobilon Chemiluminescent Substrate (Millipore) before imaging with the Bio-Rad ChemiDoc MP Imaging System (Bio-Rad).

To reprobe the membranes for a protein loading control (β -actin), all membranes were washed and placed in stripping buffer (62.5 mM Tris-HCl pH 6.7, 2% SDS, 100 mM β -mercaptoethanol) for 1 h at 60°C . After washing, the membranes were again blocked in TBS-T/5% skim milk and incubated in 0.4 μ g/mL mouse-anti- β -actin antibodies diluted in TBS-T/1% skim milk overnight at 4°C . Membranes were then washed and incubated in 0.2 μ g/mL HRP-conjugated goat anti-mouse IgG (GE Healthcare) diluted in TBS-T/1% skim milk for 2 h at RT. The remaining washing and imaging was the same as described previously.

Densitometric analysis was performed by calculating the intensity volume using the Volume Analysis Tool (Bio-Rad imaging software, Bio-Rad) and normalised to β -actin. Statistical analysis was performed using GraphPad Prism software (GraphPad Software). Changes in the abundance of paxillin and talin between normal and OH pregnancy on each day of pregnancy studied were analysed using a two-tailed Student's *t*-test. Changes in abundance of integrin $\beta 1$ and Y397FAK were

Figure 6 Protein analysis from isolated UECs showing integrin $\beta 1$ protein at 250, 130 and 115 kDa. Total integrin $\beta 1$ showed a significant decrease in the amount of total protein from day 1 of normal pregnancy compared with both day 1 of OH pregnancy and day 6 of normal pregnancy. There is a similar significant decrease in the 250 kDa band in days 6 of normal and OH pregnancy compared with day 1 of normal pregnancy. The 130 kDa band also shows a significant decrease on day 1 of OH pregnancy and day 6 of normal pregnancy compared with day 1 of normal pregnancy. There is also a significant increase in the 130 kDa isoform on day 6 of OH compared with the same time during normal pregnancy. No significant differences were detected in relation to the 115 kDa band.

Figure 7 Immunofluorescence micrographs showing the location of phosphorylated FAK (Y397FAK) on days 1 and 6 of normal pregnancy and after OH. Basal staining (arrowhead) is seen in the luminal epithelial cells (e) on days 1 of normal pregnancy as well as on days 1 and 6 of OH pregnancy. On day 6 of normal pregnancy, there is a small amount of apical immunofluorescent staining (arrow). Western blot analysis of isolated UECs shows Y397FAK as 135 kDa and 90 kDa bands with no significant difference between days or treatment groups (normal and OH pregnancy). Green staining, Y397FAK; blue, nuclei; scale bar = 20 µm.

analysed using two-way analysis of variance (ANOVA) with day of pregnancy (day 1 and day 6) and OH status (normal pregnancy and OH pregnancy) as factors. Tukey's post hoc test for multiple comparisons was then applied to determine which pairs of means were significantly different. Differences were determined to be statistically significant when $P < 0.05$. Graphs were generated using GraphPad Prism software, and data are represented as mean $\pm$ S.E.M.

Transmission electron microscopy

Uteri from 5 animals from both days 1 and 6 of normal and OH pregnancy were processed for TEM to show the ultrastructural changes. Tissue was cut into 5 mm pieces and immediately

immersed in Karnovsky's fixative (2.5% glutaraldehyde (ProSciTech, Australia), 2% paraformaldehyde (ProSciTech) in 0.1 M Sorenson's phosphate buffer (PB, pH 7.2)) for 45 min at room temperature. After cutting into 0.5–1 mm slices, tissue was returned to fresh fixative for a further 45 min. Tissue was then washed in 0.1 M PB and postfixed in 1% osmium tetroxide/0.8% potassium ferricyanide for 1 h followed by a milliQ water rinse and a 10-min 2% OsO₄ incubation. After a further rinse in milliQ water, tissue was dehydrated in a graded series of ethanol, infiltrated and embedded in Spurr's Resin in BEEM capsules (ProSciTech, Australia). Two randomly selected blocks per animal were cut using a Leica Ultracut T ultramicrotome (Leica, Heerbrugg, Switzerland), and 65–70 nm sections were mounted onto 400-mesh copper grids. Sections were post-stained with a saturated solution of uranyl acetate in 50% ethanol for 45 min, followed by Reynold's lead citrate for 10 min. Sections were viewed with a Jeol 1011 transmission electron microscope (Jeol Ltd., Japan) at 80 kV. Images were captured with a Gatan SC200 Orius CCD Camera (Gatan Inc., USA).

Results

Paxillin

On day 1 of normal pregnancy and after OH treatment (Fig. 1), paxillin is seen in the basal plasma membrane of UECs. There is very little paxillin staining on day 3 of pregnancy with and without OH stimulation (Fig. 1). However, on day 6 of pregnancy, there is a difference in paxillin staining after OH treatment (Fig. 2). In normal pregnancy on day 6, paxillin has disappeared from the basal membrane, whereas a distinct basal band is seen at this time after OH. Protein from isolated UECs shows a significant decrease in full-size paxillin ($P = 0.006$) and a significant increase in paxillin δ in OH compared with those in normal pregnancy ($P = 0.0009$). On day 7 of pregnancy (Fig. 2), basal paxillin staining reappears in UECs during normal pregnancy and is also present on day 7 of OH pregnancy. Paxillin δ is significantly increased in OH7 compared with that in D7 ($P = 0.019$).

Talin

Basal staining for talin is seen in all days of normal and OH pregnancy studied (Figs 3 and 4). Western blotting analysis of isolated uterine epithelial cells demonstrated 225 kDa and 190 kDa bands, the expected band size for talin. There was no significant change in total talin abundance in UECs between normal and OH pregnancy in any day studied (Figs 3 and 4).

Integrin $\beta 1$

Basal integrin $\beta 1$ staining is seen on day 1 of normal pregnancy and days 1 and 6 after OH (Fig. 5). On day 6 of normal pregnancy, there is integrin $\beta 1$ staining in the stroma but very little epithelial staining.

Figure 8 Electron micrographs of uterus showing the ultrastructural morphology of the basal region of luminal uterine epithelial cells (e) and underlying stroma (S) on days 1 and 6 of normal and OH pregnancy. On day 6 of normal pregnancy, the basal plasma membrane (arrow) is thickened and highly tortuous. There are no signs of basal plasma membrane thickenings. On day 1 of normal pregnancy and days 1 and 6 of OH pregnancy, the basal plasma membrane (arrow) is flattened. In higher magnification images, there are numerous basal plasma membrane thickenings, which are consistent with the morphological appearance of focal adhesions (*) (Shion & Murphy 1995). Scale bar in low mag images = 2 μ m; high mag images = 200 nm.

There is colocalisation of talin and integrin $\beta 1$ on day 1 of normal pregnancy as well as on days 1 and 6 after OH (Fig. 5). Western blot analysis of isolated luminal epithelial cells shows 250 kDa, 130 kDa and 115 kDa bands (Fig. 6). There is a significant increase in total integrin $\beta 1$ protein on day 1 of normal pregnancy compared with day 1 after OH ($P=0.0086$) and day 6 of normal pregnancy ($P=0.0067$). The larger band, 250 kDa, is significantly decreased on day 6 of normal ($P=0.0279$) and OH pregnancy ($P=0.0243$) compared with day 1 of normal pregnancy. There are also significant differences in the 130 kDa band between days 1 of normal and after OH pregnancy ($P=0.011$), between days 1 and 6 of normal pregnancy ($P=0.003$) as well as a significant increase on day 6 of OH pregnancy compared with the same time during normal pregnancy ($P=0.0284$). There were no significant differences between days and treatments in relation to the 115 kDa band.

Phosphorylated focal adhesion kinase (Y397FAK)

Y397FAK is seen basally in uterine epithelial cells on days 1 and 6 of OH pregnancy and on day 1 of normal pregnancy. There is no basal staining on day 6 of normal pregnancy but some apical staining. Western blot analysis of isolated luminal epithelial cells reveals 135 kDa and 90 kDa bands, and there are no significant differences in total protein or the individual bands between treatment groups or days (Fig. 7).

Ultrastructural morphology

Electron micrographs show a flattened basal plasma membrane on day 1 of normal and OH pregnancy (Fig. 8, arrow) with numerous thickenings in the basal plasma membrane which are morphological focal adhesions (Fig. 8, asterisks). On day 6 of normal pregnancy, the basal plasma membrane is tortuous (Fig. 8, arrow) with a thick and prominent basal lamina. However, on day 6 after OH, the basal plasma membrane is flattened (Fig. 8, arrow) with numerous morphological focal adhesions (Fig. 8, asterisks).

Figure 9 Immunofluorescent micrographs of rat uterus from day 1 of normal and OH pregnancy when incubated with normal mouse IgGs. No staining is seen, nuclei are counterstained blue. Scale bar = 20 μ m.

Controls

Non-immune controls were performed alongside all experimental runs and showed no staining. Normal and OH day 1 stained with mouse IgGs are shown as a representative example (Fig. 9).

Discussion

This is the first study to show an alteration in the morphological appearance of the basal plasma membrane, as well as the localisation and quantity of focal adhesion-associated proteins paxillin, talin, integrin $\beta 1$ and phosphorylated FAK (Y397FAK) in UECs during early pregnancy after ovarian hyperstimulation. The basal plasma membrane was found to be flattened and retains morphological focal adhesions at the time of implantation during ovarian hyperstimulated pregnancy. There was significantly less full-length paxillin and a greater abundance of paxillin δ at the time of implantation in OH compared with normal pregnancy, as well as retention of integrin $\beta 1$ and Y397FAK in the basal region of luminal uterine epithelial cells. Talin localisation and quantity, as well as the quantity of Y397FAK remained unchanged between days and OH treatment.

Retention of a flattened basal plasma membrane at the time of implantation during ovarian hyperstimulated pregnancy

During normal pregnancy in the rat at the time of implantation, the basal plasma membrane becomes highly tortuous and the basal lamina thickens (Shion & Murphy 1995). There is also a loss of morphological focal adhesions at this time (Shion & Murphy 1995). In contrast, this study found that during OH pregnancy, the basal plasma membrane remained flattened at the time of implantation, similar to the morphological appearance seen at the time of fertilisation (Shion & Murphy 1995). Furthermore, at the time of implantation in OH rats, numerous morphological focal adhesions were seen. This suggests that the UECs are more adherent to the underlying stromal tissue at the time of implantation during OH pregnancy.

Focal adhesion-associated proteins are still present at the time of implantation in OH pregnancy

This study also demonstrated the presence of paxillin, talin, integrin $\beta 1$ and Y397FAK in the basal region of UECs at the time of fertilisation in OH and normal pregnancy, as well as at the time of implantation in OH pregnancy only. This demonstrates the presence of FA-associated proteins in the same subcellular location as the morphological focal adhesions during the time of implantation in OH pregnancy. Resolution differences between light and transmission electron microscopy

would explain the appearance of an unbroken staining region of paxillin, talin, integrin $\beta 1$ or Y397FAK in the light microscope, whereas individual FA complexes are resolved using TEM.

The distinct basal localisation of paxillin seen in the immunofluorescent images on day 6 of OH pregnancy most likely represents the large pool of the paxillin δ isoform as evidenced by the significantly greater amount of this isoform compared with the same time during normal pregnancy. The larger abundance of full-length paxillin seen at the time of implantation in normal pregnancy compared with OH pregnancy seen in the Western blot data may constitute a diffuse cytoplasmic dispersion of this protein, which is unable to be detected via immunofluorescent localisation.

This alteration in the quantity of paxillin at the time of implantation between OH and normal pregnancy with less of the full-length paxillin and more paxillin δ , further demonstrates the morphological appearance and functional role of focal adhesions at this time.

Increased basal adhesion of UECs at the time of implantation in OH pregnancy

Paxillin δ , a 46kDa internal translation product of paxillin (Brown & Turner 2004, Tumbarello *et al.* 2005), increases at the time of implantation in OH compared with normal pregnancy. However, at the time of implantation during normal pregnancy, there is a significant decrease in paxillin δ compared with the time of fertilisation (Kaneko *et al.* 2008). As paxillin δ is associated with a less-motile phenotype (Sorenson & Sheibani 1999, Tumbarello *et al.* 2005), this suggests that the UECs are more firmly adherent to the underlying connective tissue at the time of implantation in OH compared with normal pregnancy.

Furthermore, this study found the colocalisation between integrin $\beta 1$ and talin at the time of implantation during OH pregnancy supporting the idea of a functional FA complex and increased basal adhesion at this time.

Results from this study suggest an increased adhesiveness between the UECs and underlying connective tissue at the time of implantation in OH pregnancy compared with the same time of normal pregnancy. The ability of the UECs to become less adherent at the time of implantation is essential for the removal of the epithelial layer to allow the blastocyst to invade into the underlying stroma and the subsequent establishment of a placenta. The retention of focal adhesions during OH pregnancy indicates that adherent UECs may provide more of a barrier to implantation of the blastocyst.

Alterations in integrins during OH pregnancy

Integrin $\beta 1$ was found in the basal region of uterine epithelial cells at the time of implantation during

OH pregnancy. The loss of integrin $\beta 1$ at the time of implantation during normal pregnancy and a decrease in the 130kDa band (expected band size) compared with day 1 of pregnancy confirms results from a previous study (Kaneko *et al.* 2011). The 250kDa dimer was found to significantly decrease on day 6 of normal and OH pregnancy compared with day 1 of normal pregnancy. The appearance of integrins as a dimer suggests an interaction within a FA complex where integrin α and β usually tightly interact (Takagi *et al.* 2001, Mouguelar *et al.* 2011). There was also an increase in the 130kDa band, the expected band size, at the time of implantation in OH pregnancy compared with normal pregnancy. This increase in monomeric integrin $\beta 1$ suggests an intact FA complex that could prevent the disassembly of Fas, which is an important process in allowing the uterine epithelial cells to become less adherent to the underlying basal lamina (Kaneko *et al.* 2008).

Phosphorylated FAK (Y397FAK) was found for the first time to be basally located within uterine epithelial cells at the time of fertilisation during normal and OH pregnancy as well as at the time of implantation during OH pregnancy. At the time of implantation during normal pregnancy, Y397FAK was only found apically where it may play a role in regulating integrin $\beta 3$ binding between the apical surface of uterine epithelial cells and trophoblastic cells of the blastocyst (Kaneko *et al.* 2011). Previous studies have shown a decrease in integrin $\beta 3$ localisation within uterine epithelial cells at the time of implantation during COH (Biyiksiz *et al.* 2011). Although there was no change in Y397FAK protein quantity between OH and normal pregnancy at the time of implantation, there was a very different localisation. During OH pregnancy, Y397FAK remained localised to the basal region of uterine epithelial cells where it is proposed to be associated with the FA complex. This is also the time when integrin $\beta 1$ and talin are colocalised, suggesting a stable FA complex.

Ovarian hormones and retention of focal adhesions at the time of implantation in OH pregnancy

Previous studies using this rat OH model have found an alteration in ovarian hormones oestradiol and progesterone in OH compared with that in normal pregnancy (Jovanovic & Kramer 2010) with an increase in the progesterone:oestradiol ratio observed at implantation. This decrease in oestradiol and increase in progesterone seen at this time does not fully explain the distributional and quantitative changes observed in the focal adhesion-associated proteins seen in this study. The flattening of the basal plasma membrane as well as the increase in paxillin δ seen at the time of implantation in OH compared with normal pregnancy is also observed in ovariectomised rats treated with 3 days of oestradiol treatment, but not in animals treated with progesterone alone or in combination with oestradiol

(Shion & Murphy 1995, Kaneko *et al.* 2009). Similarly, the basal localisation of integrin $\beta 1$ and total FAK was also seen in oestradiol-treated ovariectomised animals (Kaneko *et al.* 2011, 2012). Thus, although there is an alteration in ovarian hormones during OH pregnancy, these are not sufficient to explain the changes in key focal adhesion molecules seen in this study at the time of implantation in OH pregnancy.

The use of animal models of ovarian hyperstimulated pregnancy has already demonstrated disturbances in uterine fluid transport mechanisms (Lindsay & Murphy 2014), alterations in adhesion molecules (Sendag *et al.* 2010, Biyiksiz *et al.* 2011) and now changes in the components of key basal structural complexes, which indicate that UECs may be less easily removed by an implanting blastocyst. A picture of a mechanistic change in UECs in response to OH therapy is becoming evident, and this may have wider implications for human IVF cycles. More studies are required to investigate if similar morphological changes occur in response to COH in humans.

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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