ITGAV (alpha v integrins) bind SPP1 (osteopontin) to support trophoblast cell adhesion

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Abstract

Attachment of the conceptus trophoblast (Tr) to the uterine luminal epithelium (LE) is critical for successful implantation. This study determined whether alpha v (av) integrins (ITGAV) directly mediate porcine trophoblast cell adhesion to secreted phosphoprotein 1 (SPP1, also known as osteopontin (OPN)) and examined the temporal/spatial expression of ITGAV, beta 3 (b3, ITGB3) and beta 6 (b6, ITGB6) integrin subunits, and SPP1, at the uterine-placental interface of pigs. Knockdown of *ITGAV* in porcine Tr (pTr2) cells by siRNA reduced pTr2 attachment to SPP1. *In situ* hybridization confirmed the presence of *ITGAV*, *ITGB3* and *ITGB6* mRNAs in uterine LE and conceptus Tr between Days 9 and 60 of gestation, with no change in the magnitude of expression over the course of pregnancy. Exogenous E2 or P4 did not affect *ITGAV*, *ITGB3* and *ITGB6* mRNA expression in the uteri of ovariectomized gilts. Immunofluorescence identified ITGAV, ITGB3 and SPP1 proteins in large aggregates at the uterine LE-placental Tr/chorion interface on Day 25, but aggregates were no longer observed by Day 50 of gestation. These results are the first to directly demonstrate that pTr2 cells engage ITGAV-containing integrin receptors to adhere to SPP1 and suggest that mechanical forces generated by tethering elongating conceptuses to uterine LE leads to assembly of focal adhesions containing ITGAV and SPP1; however, as placentation progresses, subsequent folding/interdigitation at the uterine-placental interface disperses mechanical forces resulting in the loss of focal adhesions.

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Introduction

Integrins are heterodimeric transmembrane receptors, formed from 18 alpha (a)- and 8 beta (b)-subunits, capable of dimerizing to form 24 different heterodimer combinations that can bind to numerous extracellular ligands including a variety of extracellular matrix (ECM) proteins (Albelda & Buck 1990, Gallant *et al.* 2005, Humphries *et al.* 2006). Of the 18 alpha integrin subunits, alpha v (av, ITGAV) combines with the greatest number of beta subunits to form the $\alpha v\beta 1$ (ITGAVB1), $\alpha v\beta 3$ (ITGACB3), $\alpha v\beta 5$ (ITGAB5), $\alpha v\beta 6$ (ITGAVB6) and $\alpha v\beta 8$ (ITGAVB8) integrin receptors (Humphries *et al.* 2006). Integrins are dominant glycoproteins in many cell adhesion cascades, including well-defined roles in leukocyte adhesion to the apical surface of polarized endothelia for extravasation of leukocytes from the vasculature into tissues (Kling *et al.* 1992). A similar adhesion cascade involving apically expressed integrin receptors on uterine luminal epithelium (LE) and conceptus trophoblast (Tr) is proposed as a mechanism for initial attachment of conceptus Tr to uterine LE for implantation (Denker 1993, Aplin *et al.* 1994, Burghardt *et al.* 2002). The general consensus is

that integrins of the ITGAV family are involved in this attachment (Aplin 1997), and, because secreted phosphoprotein 1 (SPP1, also known as osteopontin (OPN)) expression increases markedly in uterine LE during the peri-implantation period of pigs, sheep, goats, humans, rabbits and mice (Johnson *et al.* 1999b, Garlow *et al.* 2002, Apparao *et al.* 2003, Mirkin *et al.* 2005, Joyce *et al.* 2005a, White *et al.* 2006), SPP1 is considered to be an excellent candidate adhesion ligand for ITGAV during implantation (Johnson *et al.* 2003, 2014).

The ITGAV subunit has been associated with the peri-implantation period of mammals since the early 1990s. Lessey and coworkers established that transient uterine expression of ITGAVB3 during the menstrual cycle and early pregnancy defines the 'window of implantation' in women (Lessey *et al.* 1994, 1996). Null mutation of the *Itgav* gene in mice leads to peri-implantation lethality (Hynes 1996), whereas functional blockade of ITGAV, using neutralizing antibody, reduces the number of implantation sites in mice and rabbits (Illera *et al.* 2000, 2003). The ITGAV subunit has also been localized to the apical surface of uterine LE of species

that have non-invasive implantation, including pigs, sheep, goats and cattle (Bowen *et al.* 1996, Guillomot 1999, Johnson *et al.* 1999b, Kimmins & MacLaren 1999). Indeed, in pigs, co-localization of ITGAV and talin (TLN) at the apical domains of conceptus Tr and uterine LE cells of the uterine-placental interface strongly suggests an active role for ITGAV in the formation of focal adhesion (FA)-mediated attachments during the peri-implantation period of pregnancy. Erikson and coworkers demonstrated the clustering and aggregation coalescence of ITGAV into structures interpreted to be *in vivo* focal adhesions at the apical surface of a pig Tr cells on Day 20 of gestation, when firm attachment of conceptus Tr to LE is established, suggesting further investigation of ITGAV in porcine conceptus Tr cells was warranted (Erikson *et al.* 2009). Studies using affinity chromatography followed by immunoprecipitation have shown that SPP1 binds the ITGAV and ITGB6 subunits on porcine conceptus Tr (pTr2) cells and the ITGAV and ITGB3 integrin subunits on cultured porcine uterine LE cells and that SPP1 promotes dose- and integrin-dependent attachment of pTr2 to cells (Erikson *et al.* 2009). Further, porcine pTr2 cells cultured with SPP1-coated microspheres revealed co-localization of the ITGAV subunit and TLN to FAs at the apical domain of these cells (Erikson *et al.* 2009). These results indicate that SPP1 binds the ITGAV subunit on pTr2 cells and that SPP1 supports pTr2 cell adhesion, but they do not show that adhesion of pTr2 cells to SPP1 directly involves the ITGAV.

In pigs, *SPP1* mRNA is induced by conceptus estrogens in discrete regions of the uterine LE juxtaposed to the conceptus on Day 13, just prior to implantation, and is abundant in the entire uterine LE by Day 20, when firm adhesion of conceptus Tr to uterine LE has occurred (Garlow *et al.* 2002, White *et al.* 2005). At this same interface, conceptus Tr and uterine LE express multiple integrin subunits that potentially form heterodimer receptors that bind to SPP1 (Bowen *et al.* 1996). Apical attachment between uterine LE and placental Tr/chorion cells is maintained throughout gestation in pigs, which have epitheliochorial placentation (Dantzer 1984), and uterine LE cells continue to express *SPP1* mRNA at the uterine-placental interface (Garlow *et al.* 2002). It is reasonable to predict that integrins and SPP1 continue to attach the placental chorion to the uterine LE in pigs; however, at present, there is no information about the localization of SPP1 protein or mRNAs and proteins for the integrins hypothesized to mediate this attachment. Therefore, to advance our understanding of the role(s) of ITGAV and SPP1 during implantation and placentation in pigs, the current study was performed to mechanistically determine whether SPP1 binds ITGAV-containing integrins to directly support the adhesion of pTr2 cells *in vitro*, and to establish the temporal, cell-type-specific expression and hormonal regulation

of the ITGAV subunit, its counterparts the ITGB3 and ITGB6 subunits and SPP1 at the uterine-placental interface of pigs throughout gestation.

Materials and methods

Porcine cell culture

The porcine Tr cell line (pTr2) was developed from conceptuses collected on Day 12 of gestation and propagated as described previously (Ka *et al.* 2001) in Dulbecco modified Eagle medium-Ham F12 (DMEM-F12; Sigma-Aldrich) with 10% (vol/vol) fetal bovine serum (FBS). Cells were maintained in a 5% CO₂ humidified environment at 37°C.

RNA isolation and RT-PCR analyses

Total cellular RNA was isolated from pTr2 cells using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Complementary DNA was prepared using 3 µg total RNA with the SuperScript III First-Strand Synthesis System (Life Technologies) according to the manufacturer's instructions. After cDNA synthesis, PCR was performed using 50 ng/reaction of cDNA with a recombinant Taq DNA polymerase (Life Technologies) according to the manufacturer's instructions. Primers for integrin subunits *ITGA5*, *ITGAV*, *ITGA2*, *ITGA4*, *ITGB1*, *ITGB3*, *ITGB6* and *GAPDH* (positive control; Supplementary Table 2, see section on supplementary data given at the end of this article) were derived from conserved porcine sequences and designed using Primer3 (primer3.sourceforge.net/) and produced by Eurofins MWF Operon (Huntsville, AL). The PCR products were analyzed on 1% (wt/vol) agarose gels, and the gels were imaged using the PhotoDoc-It imaging system (UVP, Upland, CA).

Knockdown of ITGAV in pTr2 cells

The pTr2 cells were grown to confluence in T75 flasks prior to transfection. The siRNAs were designed (Supplementary Table 3) from established sequences for porcine *ITGAV* and *GAPDH* and produced by Ambion. Individual mixtures with siRNA were assembled by combining 30 µL siPortAmine (Ambion) in 3.482 mL OPTI-MEM (Life Technologies). Cells were plated at 80% confluence and transfected with 50 nM siRNA in antibiotic-free DMEM containing 10% FBS. Treatment groups included vehicle, *ITGAV* siRNA or *GAPDH* siRNA. At 8 h, cells were supplemented with 15 mL DMEM-F12 with 10% FBS. The medium was changed at 24 h, and the cells were grown to confluence for 72 additional hours prior to use. Adhesion experiments were performed with 4 intra-assay replicates per treatment per siRNA and were performed three times to ensure the statistical viability. Prior to utilizing knockdown cells for assays, three different clones of each siRNA (provided by Ambion) were tested at concentrations of 10, 50 or 100 nM. The optimal clone (sequence noted in Table 3) was utilized at a concentration of 50 nM as it produced the optimal knockdown efficiency.

Western blot analyses

Western blots were performed on protein extracts of pTr2 cells from each siRNA treatment group using RIPA lysis buffer (50 mM Tris-HCl, 150 mM Triton X100, 1.0% sodium deoxycholate w/v, 0.1% SDS w/v, 100 mM Na_3VO_4) containing complete EDTA-free protease inhibitors (Roche Diagnostics). Cells were pelleted and flash frozen on dry ice and stored at -80°C until extraction was performed. For protein extraction, cells were re-suspended in RIPA lysis buffer and incubated on ice for 10 min. Cells were then sheared by pipetting multiple times. Protein concentrations were determined using a modified Lowry assay (Cat# 500-0116, Bio-Rad) on a NanoDrop 1000 spectrophotometer (Thermo Scientific). Laemmli sample buffer was added to protein samples to a $1\times$ final concentration (50 mM Tris-Cl, 2% wt/vol SDS, 0.1% wt/vol bromophenol blue, and 10% vol/vol glycerol) and samples were heated at 95°C for 5 min. Proteins were separated by 10% SDS-polyacrylamide gels (100 $\mu\text{g}/\text{lane}$) and transferred to nitrocellulose membranes (0.45 μm , GE Healthcare). Immunoreactive proteins were detected as described previously (Joyce *et al.* 2005b). Briefly, membranes were washed with Tris-buffered saline-Tween20 (TBST; 20 mM Tris base, 137 mM NaCl and 0.1% Tween 20; pH 7.6) and blocked with 5% milk protein in TBST for 1 h at room temperature. Rabbit anti-human αv integrin immunoglobulin (1 mg/mL), mouse anti-rabbit GAPDH immunoglobulin (1 mg/mL, ab8245, Abcam) or mouse anti-human cytokeratin immunoglobulin (1 mg/mL, Sigma-Aldrich) were diluted 1:1000, 1:1000 and 1:3000 in blocking solution respectively and incubated on blots with constant agitation overnight at 4°C . Blots were then washed again and probed with either goat anti-rabbit HRP immunoglobulin or goat anti-mouse HRP immunoglobulin (1 mg/mL, 474–1506 and 474–1806, KPL, Gaithersburg, MD, USA) diluted 1:5000 in 5% milk protein in TBST and incubated for 1 h at room temperature followed by washing with TBST. Blots were developed with the Pierce SuperSignal West Pico chemiluminescence substrate detection system (Thermo Scientific) according to the manufacturer's instructions. Multiple exposures were generated to ensure the linearity of film exposures. Blots were stripped as needed with Restore Western blot stripping solution between probes. Blots were first probed with rabbit anti-human αv immunoglobulin or mouse anti-rabbit GAPDH immunoglobulin, and then stripped and re-probed with mouse anti-human cytokeratin immunoglobulin.

Cell adhesion analyses

Cell adhesion assays were conducted as previously described (Bayless *et al.* 2009, Erikson *et al.* 2009). High-binding polystyrene microwells (Corning-Costar, Sigma-Aldrich) were coated overnight at 4°C with 50 μL of 20 $\mu\text{g}/\text{mL}$ of bovine milk SPP1, bovine fibronectin (FN), bovine serum albumin (BSA; negative control) and type I collagen (COL1A1; positive control; $n=4$ replicates/treatment) in PBS. After blocking each well in 10 mg/mL BSA in PBS (100 μL), 50,000 pTr2 cells were added per well and allowed to attach for 1 h (37°C , 5% CO_2). In all cell attachment experiments, nonadherent cells were removed by washing in isotonic saline and attached cells were fixed in 4% vol/vol formalin in PBS. Plates were stained with

0.1% wt/vol amido black in 30% methanol and 10% acetic acid for 15 min, rinsed and solubilized with 2 N NaOH (50 μL per well) to obtain an absorbance reading at 595 nm, which directly correlated with the number of cells stained in each well. Background (BSA) absorbance values were subtracted from treatment absorbance, and a two-way ANOVA with a Bonferroni post-test was utilized to determine statistical significance with $P<0.05$ considered significant.

Immunofluorescence analyses of cultured cells

The 15 mm coverslips used in this analysis were coated with recombinant rat SPP1 (20 $\mu\text{g}/\text{mL}$) overnight at 4°C . pTr2 cells (1.5×10^5) were seeded onto the coverslips and allowed to attach for 2 h (37°C , 5% CO_2) in DMEM-F12. Cells were then fixed with 4% paraformaldehyde for 10 min and washed 2 times with glutaraldehyde. Immunofluorescence staining was performed as previously described (Johnson *et al.* 2001). After washing with PBS containing 0.3% vol/vol Tween20, cells were blocked with 10% vol/vol goat serum and incubated overnight at 4°C with a monoclonal antibody to human αv integrin (1 mg/mL, AB1930). Cell-bound primary antibody was then detected with goat anti-rabbit IgG Alexa 488 (1 mg/mL, Life Technologies). Mouse immunoglobulin at the same concentration as the primary monoclonal antibodies was used as a negative control. Coverslips were adhered to corning pre-cleaned single-frosted slides via Prolong Antifade Mounting Reagent with DAPI (Invitrogen).

Animals and tissue collection

All experimental and surgical procedures complied with the Guide for Care and Use of Agriculture Animals and were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University. Sexually mature gilts were observed for signs of estrus (Day 0 (estrus/mating)) daily and exhibited at least two estrous cycles of normal duration (18–21 days) prior to being used in these studies.

To assess the effects of pregnancy on the temporal and spatial expression of integrin subunits, gilts were assigned randomly to either cyclic or pregnant status. Gilts in the cyclic group were hysterectomized on Day 9, 12 or 15 of the estrous cycle. Gilts assigned to the pregnant group were naturally bred to reproductively competent boars and hysterectomized on Day 9, 12, 13, 14, 15, 20, 24, 25, 30, 35, 40, 45, 50 or 60 of pregnancy ($n=4$ pigs/day/status).

To assess the effects of estrogen on the expression of integrins by the uterine epithelium, a previously validated pseudopregnancy model was utilized (White *et al.* 2005). Gilts were randomly assigned to pseudopregnant or control status. Gilts for pseudopregnancy received intramuscular injections of estradiol benzoate (5 mg in 5 mL of corn oil/day) or corn oil alone ($n=4/\text{treatment}$) on Days 11, 12, 13 and 14 of the estrous cycle. All gilts were then hysterectomized on Day 15 of the estrous cycle or pseudopregnancy.

To evaluate the effects of long-term progesterone on the expression of integrins by the uterine epithelium, gilts were treated with progesterone as previously described (Bailey *et al.* 2010). Gilts were ovariectomized on Day 12 of the estrous

cycle and assigned randomly to receive daily injections (i.m.) of either 4 mL corn oil or 200 mg P4 in 4 mL corn oil on Days 12–39 post-estrus ($n=4/\text{treatment}$). All gilts were hysterectomized on Day 40 post-estrus.

To assess distribution of integrin subunit mRNAs, at hysterectomy, several cross sections (approximately 1–1.5 cm thick) from the middle of each uterine horn from all gilts were placed in fresh 4% paraformaldehyde fixative for 24 h and then embedded in Paraplast Plus (Oxford Labware, St Louis, MO, USA). To assess the assembly of FAs at the uterine–placental interface that incorporate integrin subunit proteins, several 1–1.5 cm sections of uterine wall from the middle of each uterine horn of all pregnant gilts were snap frozen in Tissue-Tek OCT compound (Miles, Oneonta, NY, USA).

Cloning of partial cDNAs for porcine *ITGAV*, *ITGB3* and *ITGB6*

Partial cDNAs for *ITGAV*, *ITGB3* and *ITGB6* integrin subunits were generated by RT-PCR as previously described using total RNA from endometrial tissue from Day 25 pregnant gilts (Joyce *et al.* 2008). Primers for each target gene (Supplementary Table 1) were designed from sequences of the respective porcine genes using Primer 3 (Version 2.0-alpha; <http://primer3.sourceforge.net/>). PCR amplification was conducted as follows for porcine *ITGAV*, *ITGB3* and *ITGB6* subunits: (1) 95°C for 5 min; (2) 95°C for 45 s, 60.4°C (*ITGAV*) or 62.4°C (*ITGB3* and *ITGB6*) and 72°C for 1 min for 35 cycles and 3) 72°C for 10 min. Amplified PCR products were subcloned into the pCRII cloning vector using a TOPO TA Cloning kit (Invitrogen) and sequenced in both directions using an ABI PRISM Dye Terminator Cycle Sequencing kit and an ABI PRISM automated DNA sequencer (Applied Biosystems) to confirm sequence identity.

***In situ* hybridization analyses**

ITGAV, *ITGB3* and *ITGB6* mRNAs were localized in paraffin-embedded uterine and placental tissues as previously described (Johnson *et al.* 1999a). Uterine cross-sections from each animal (5 µm) were deparaffinized, rehydrated and deproteinized and then hybridized with radiolabeled antisense and sense cRNA probes generated from linearized plasmid DNA templates. Radiolabeled antisense or sense cRNA probes were synthesized by *in vitro* transcription with [α -³⁵S] uridine 5-triphosphate (PerkinElmer Life Sciences). After hybridization, washing and RNase A digestion were performed, followed by autoradiography using NTB-2 liquid photographic emulsion (Eastman Kodak). Slides were exposed at 4°C for 8 days, developed in Kodak D-19 developer, counterstained with Harris-modified hematoxylin (Thermo Fisher Scientific), dehydrated and protected with cover slips sealed with Permount (Fisher Scientific).

Quantification of *in situ* hybridization

To quantify mRNA levels observed on *in situ* hybridization slides, three digital photomicrographs were taken per slide/day of pregnancy or cycle using an AxioPlan 2 microscope

(Carl Zeiss). Photomicrographs were captured as tagged image file (TIF) and loaded into Adobe Photoshop (version 9.0, Adobe Systems) to measure the intensity of silver grains. Six sections across the uterine–placental interface were chosen at random to measure the integrated density and area was measured. These measurements were then analyzed and graphed as a ratio of density/area. A one-way ANOVA was utilized to determine the statistical significance with $P<0.05$ considered significant.

Immunofluorescence analyses of uterine and placental tissues

SPP1, *ITGAV* and *ITGB3* proteins were localized in frozen uterine cross sections (Days 24, 25, 50, 70 and 90 of pregnancy, $n=4/\text{day}$) as previously described (Johnson *et al.* 2001). Briefly, tissues were fixed in –20°C methanol, rinsed in PBS containing 3% (vol/vol) Tween20, blocked in 10% (vol/vol) normal goat serum and incubated overnight with 2 µg/mL of rabbit antiserum directed against human anti- α subunit, human anti- β 3 subunit or human anti-SPP1 (AB1930, AB1968, AB10910; EMD Millipore International). Tissue-bound primary antibody was detected with goat anti-rabbit IgG Alexa 488 (8 µg/mL). Slides were overlaid with Prolong Gold Antifade reagent with DAPI (Life Technologies) and a cover glass.

Photomicrography

Digital photomicrographs of *in situ* hybridization (brightfield and darkfield images) and immunofluorescence staining were evaluated using an AxioPlan 2 microscope (Carl Zeiss) interfaced with an AxioPlan HR digital camera and AxioVision 4.3 software. Individual fluorophore images (Alexa 488 and DAPI) were recorded sequentially with AxioVision 4.3 software and evaluated in multiple fluorophore overlay images recorded in Zeiss Vision Image (ZVI) file format, which were subsequently converted to tagged image file (TIF) format. Photographic plates were assembled using Adobe Photoshop (version 6.0, Adobe Systems). All sections from each day/treatment were assessed as a group and sections exhibiting the most representative hybridization/immunostaining pattern for each day/treatment were selected for inclusion in photographic plates.

Results

Characterization of integrin subunits present in pTr2 cells

Integrin subunits expressed by pTr2 cells were characterized using PCR analyses and revealed the presence of integrin subunits *ITGAV*, *ITGA2*, *ITGA5*, *ITGB1*, *ITGB3* and *ITGB6*, but not *ITGA4* (Fig. 1A). Detection of *GAPDH* mRNA was used as a positive control for the PCR reaction. Based on these data, the schematic in Fig. 1B depicts the integrin heterodimeric protein receptors that are potentially present on pTr2 cells. There are four potential receptors that bind SPP1 ($\alpha 5\beta 1$ (*ITGA5B1*), *ITGAVB3*, *ITGAVB6* and *ITGAVB1*),

Figure 1 Expression of integrin subunit mRNAs by the porcine trophoblast (pTr2) cell line. (A) PCR profile of integrin subunits (labeled with their traditional Greek letters) expressed by pTr2 cells. (B) Schematic depicting potential integrin heterodimeric receptors (labeled with their traditional Greek letters) present on pTr2 cells based upon PCR analysis. Schematics show integrin expression in wild-type pTr2 cells (left panel) and pTr2 cells treated with siRNA directed to the ITGAV (α_v) subunit (right panel). FN, fibronectin; KD, knockdown.

three potential receptors that bind FN (ITGA5B1, ITGAVB3 and ITGAIIB3) and one potential receptor that binds COL1A1 (ITGA2B1) (Humphries *et al.* 2006). Because the ITGAV subunit is a component of three out of four of the integrin receptors that bind SPP1, we chose to knockdown the expression of the ITGAV subunit protein in pTr2 cells using siRNA, anticipating that the loss of three integrin receptors reported to bind SPP1 (ITGAVB3, ITGAVB6 and ITGAVB1) and one integrin receptor that binds FN (ITGAVB3) would decrease the adhesion to SPP1 and FN (Fig. 1B). Because ITGAVs are not involved in binding to COL1A1, pTr2 attachment through ITGA2B1 was still expected to occur (Fig. 1B).

Effects of ITGAV subunit knockdown in pTr2 cells

Western blotting was utilized to determine the ability of siRNAs to knockdown ITGAV protein in pTr2 cells. Cells were treated with no siRNA (Control) or siRNAs targeting either integrin *ITGAV* or *GAPDH*. The *GAPDH* siRNA was utilized as a negative control. Cells transfected with *ITGAV* siRNA had a reduction in ITGAV protein in cell lysates when compared to the control and *GAPDH* siRNA-transfected cells (Fig. 2A). Cells treated with *GAPDH* siRNA exhibited a decrease in GAPDH protein in cell lysates compared to those in control and *ITGAV* siRNA-treated cells (Fig. 2A). Treatment with *ITGAV* or *GAPDH* siRNAs did not affect the expression of cytokeratin (CK), an epithelial cell intermediate filament in pTr2 cells (Fig. 2A). An adhesion assay was utilized to determine the effects of ITGAV subunit knockdown on the ability of pTr2 cells to adhere to FN, SPP1 or

COL1A1. There was a significant decrease in the number of pTr2 cells that adhered to FN and SPP1 in the *ITGAV* siRNA-treated group when compared to treatment with control or *GAPDH* siRNAs (Fig. 2B). Therefore, the ITGAV subunit has a direct role in the attachment of pTr2 cells to FN and SPP1. As expected, there was no difference in attachment of pTr2 cells among treatment groups to COL1A1 because COL1A1 does not bind to ITGAV-containing integrin heterodimers (Fig. 2B). Immunofluorescence was utilized to determine whether knockdown of the ITGAV subunit affects the ability of pTr2 cells to bind SPP1 and form discrete aggregates at their basal surface. Aggregates of ITGAV subunits were observed in the *GAPDH* siRNA treatment group at the periphery of the cells attached to SPP1-coated coverslips (Fig. 2C). In contrast, there were few ITGAV subunit-containing aggregates observed when pTr2 cells were transfected with *ITGAV* siRNA compared to those in the *GAPDH* siRNA treatment group (Fig. 2C). In addition, cells treated with *ITGAV* siRNA did not flatten and spread on the SPP1 when compared with the *GAPDH* siRNA-treated cells (Fig. 2C). These data indicate both pTr2 attachment and spreading on immobilized SPP1 substrate is significantly compromised with silencing ITGAV.

Temporal and spatial expression of ITGAV, ITGB3, ITGB6 and SPP1

In situ hybridization analyses localized *ITGAV*, *ITGB3* and *ITGB6* mRNAs to uterine LE at the uterine-placental interface in cyclic and pregnant animals (Figs 3, 4 and 5)

Figure 2 siRNA knockdown of ITGAV decreases the adhesion of pTr2 cells to OPN. (A) Western blot showing decreased ITGAV (αv) protein in cells treated with *ITGAV* siRNA, decreased GAPDH protein in cells treated with *GAPDH* siRNA and equal loading of gels using anti-cytokeratin IgG to detect protein. (B) Adhesion of cells to extracellular matrix molecules. pTr2 cells were added to multi-well plates that were coated with 20 μ g/mL BSA (negative control), bovine fibronectin (fibronectin), bovine milk SPP1 or collagen type 1 (collagen) for 1 h prior to fixing and staining with Amido Black and quantification of absorption at 595 nm. Error bars represent the standard error of the mean $P \leq 0.05$. (C) Immunofluorescence localization of ITGAV on pTr2 cells bound to SPP1. Immunofluorescence analysis confirmed that ITGAV is a component of FAs (white arrows) that assemble at the base of pTr2 cells adhering to a rigid substrate. However, siRNA knockdown of ITGAV protein decreased the assembly of FAs in response to binding SPP1. Samples shown in all panels A, B and C were collected and analyzed from a single, representative experiment. Images are 230 and 140 μ m wide for images obtained with 40 $\times$ and 63 $\times$ microscope objectives respectively. Antibody used to detect ITGAV (AB1930; Millipore).

respectively. Integrin mRNA was observed at the uterine-placental interface during the peri-implantation period of pregnancy on Days 20 and 25 (Figs 3A, 4A and 5A). Relative mRNA levels were quantified by measuring the density of silver grains in the uterine LE or at the uterine-placental interface (Figs 3B, 4B and 5B). No change in relative mRNA levels was observed for

Figure 3 *ITGAV* subunit mRNA in pig endometria and placenta. (A) *In situ* hybridization revealed *ITGAV* mRNA in uterine luminal epithelial (LE) and conceptus trophoderm (Tr) cells during the estrous cycle and pregnancy. Corresponding brightfield and darkfield images from Days 20 and 25 of pregnancy (P) hybridized with radiolabeled antisense RNA probe are shown. A representative section hybridized with radiolabeled sense RNA probe (sense) serves as a negative control. Width of field for all microscopic images is 940 μ m. (B) Relative density of silver grains per area measured, from sections taken from different days of the estrous cycle (C) and pregnancy was graphed and analyzed by one-way ANOVA. Hybridization for *ITGAV* mRNA did not change over days of pregnancy ($P > 0.05$).

the three integrin subunits between cyclic and pregnant gilts or between days of pregnancy. Therefore, *ITGAV*, *ITGB3* and *ITGB6* mRNAs are available for translation in conceptus Tr and uterine LE, but these integrin receptors are not upregulated on the transcriptional level while the conceptus is undergoing implantation.

Estrogen was used to induce pseudopregnancy, and its effects on integrin *ITGAV*, *ITGB3* and *ITGB6* mRNA expression were examined. *In situ* hybridization analyses revealed that uterine *ITGAV*, *ITGB3* and *ITGB6* mRNA localization was not different between control and pseudopregnant gilts (Supplementary Fig. 1). Progesterone was injected into ovariectomized gilts,

Figure 4 *ITGB3* subunit mRNA in pig endometria and placentae. (A) *In situ* hybridization revealed *ITGB3* mRNA in uterine luminal epithelial (LE) and conceptus trophoblast (Tr) cells during the estrous cycle and pregnancy. Corresponding brightfield and darkfield images from Days 20 and 25 of pregnancy (P) hybridized with radiolabeled antisense RNA probe are shown. A representative section hybridized with radiolabeled sense RNA probe (sense) serves as a negative control. Width of field for all microscopic images is 940 μ m. (B) Relative density of silver grains per area measured, from sections taken from different days of the estrous cycle (C) and pregnancy, was graphed and analyzed by one-way ANOVA. Hybridization for *ITGB3* mRNA did not change over days of pregnancy ($P > 0.05$).

and effects of progesterone treatment in the absence of estrogen on *ITGAV*, *ITGB3* and *ITGB6* mRNA expression were examined. *In situ* hybridization analyses revealed that uterine *ITGAV*, *ITGB3* and *ITGB6* mRNA localization was not different between control and progesterone-treated gilts (Supplementary Fig. 1).

Immunofluorescence was performed to determine temporal and spatial localization of *ITGAV*, *ITGB3* and *SPP1* proteins during porcine pregnancy. We were unable to identify antibodies that cross-reacted with the pig *ITGB6* protein (data not shown). The *ITGAV* and *ITGB3* subunits were present in large intermittent

Figure 5 *ITGB6* subunit mRNA in pig endometria and placentae. (A) *In situ* hybridization revealed *ITGB6* mRNA in uterine luminal epithelial (LE) and conceptus trophoblast (Tr) cells during the estrous cycle and pregnancy. Corresponding brightfield and darkfield images from Days 20 and 25 of pregnancy (P) hybridized with radiolabeled antisense RNA probe are shown. A representative section hybridized with radiolabeled sense RNA probe (sense) serves as a negative control. Width of field for all microscopic images is 940 μ m. (B) Relative density of silver grains per area measured, from sections taken from different days of the estrous cycle (C) and pregnancy was graphed and analyzed by one-way ANOVA. Hybridization for *ITGB6* mRNA did not change over days of pregnancy ($P > 0.05$).

aggregates at the uterine-placental interface on Day 25 of pregnancy (Figs 6 and 7), similar to what was reported on Day 20 in pigs (Erikson et al. 2009). These large protein aggregates were no longer observed at the uterine-placental interface in pigs by Day 50 of pregnancy and remained absent at the interface thereafter (Days 25 and 50 shown in Figs 6 and 7; Days 70 and 90 shown in Supplementary Fig. 2). Similar to the patterns of expression of *ITGAV* and *ITGB3* integrin subunits, intermittent aggregates of *SPP1* protein were present at the uterine-placental interface on Day 25, but *SPP1* protein was distributed evenly along the

Figure 6 Immunofluorescence detection of ITGAV subunit protein at the uterine-placental interface on Days 25 and 50 of pregnancy. Large aggregates of ITGAV (α V Integrin) protein were detected at the interface between uterine luminal epithelial (LE) and conceptus trophoblast/chorionic epithelial (Tr) cells on Day 25, but were not detected on Day 50 of gestation. Nuclei are stained blue with DAPI. Width of fields for images obtained with a 16 $\times$ microscope objective is 540 μ m, and 230 μ m for a 40 $\times$ objective. Unlabeled arrows indicate the interface between LE and Tr.

uterine-placental interface on Days 35 and 60 of pregnancy (Fig. 8). It is noteworthy that SPP1 protein was detected along the uterine-placental interface throughout pregnancy in pigs and in the glandular epithelium (GE) by Day 35, which was expected due to previously reported expression of *SPP1* mRNA by uterine LE and GE (Garlow *et al.* 2002). The ITGAV subunit was also present in the endometrial stroma on Days 50 and 70 of pregnancy in pigs (Fig. 6 and Supplementary Fig. 2).

Discussion

Implantation begins when the apical surfaces of uterine LE and conceptus Tr cells adhere to each other (Denker 1993, Aplin 1997, Burghardt *et al.* 2002), and in pigs, these two cell layers remain attached throughout gestation (Dantzer 1984). Our laboratory has a long-term interest in the ability of SPP1 and its integrin receptors to promote the adherence of conceptus Tr to uterine LE during implantation (Johnson *et al.* 2003, 2014). Results of this study of pigs are the first to demonstrate that (1) ITGAV directly interacts with SPP1 to adhere pTr2 cells to culture plates; (2) mRNAs for *ITGAV*, *ITGB3* and *ITGB6* are expressed in uterine LE

and conceptus Tr throughout gestation, but expression does not change and is not regulated by E2 or P4; (3) ITGAV, ITGB3 and SPP1 proteins co-localize in large aggregates at the uterine-placental interface on Day 25 of pregnancy and (4) aggregates of ITGAV and ITGB3 proteins are no longer detectable and aggregates of SPP1 protein are replaced by uniform immunostaining, at the highly folded uterine-placental interface on Days 50, 70 and 90 of gestation. Recently, Aplin and coworkers (Kang *et al.* 2014) employed three *in vitro* models of early implantation with siRNA-treated endometrial epithelial Ishikawa cells to demonstrate that ITGAVB3 receptor at the surface of these cells directly interacts with SPP1 to support the adhesion of mouse blastocysts in culture (Kang *et al.* 2014). The present study supports their findings and expands our understanding about the potential of SPP1 to bridge conceptus Tr to uterine LE by being the first to demonstrate a functional involvement, using siRNA, for ITGAV in adhesion of pTr2 cells to SPP1 (Fig. 1).

In the present study, the pTr2 cells were first characterized to determine their integrin expression profile. PCR analyses showed the presence of *ITGAV*, *ITGA2*, *ITGA5*, *ITGB1*, *ITGB3* and *ITGB6*, but not *ITGA4*

Figure 7 Immunofluorescence detection of ITGB3 integrin subunit protein at the uterine-placental interface on Days 25 and 50 of pregnancy. Large aggregates of ITGB3 ($\beta 3$ integrin) protein were detected at the interface between uterine luminal epithelial (LE) and conceptus trophoderm/chorionic epithelial (Tr) cells on Day 25 but were not detected on Day 50 of gestation. Width of fields for images obtained with a 16 $\times$ microscope objective is 54 μ m and 230 μ m for a 40 $\times$ objective. Unlabeled arrows indicate the interface between LE and Tr.

subunits, and these same subunits have been previously observed on pig conceptus Tr *in vivo* (Bowen *et al.* 1996, Erikson *et al.* 2009). To determine the physiological relevance of ITGAV in pTr2 cells, siRNA was utilized to knockdown the ITGAV subunit, and treated cells were analyzed for their ability to bind different ECM proteins. When expression of ITGAV protein was decreased in pTr2 cells, the ability of pTr2 cells to adhere to SPP1 was reduced. It is notable that in the same experiment, decreased expression of ITGAV protein also decreased pTr2 cell adhesion to FN, a matrix protein capable of binding to multiple integrins including the ITGAV $\beta 3$ heterodimer, but did not affect pTr2 adhesion to COL1A1, which does not bind ITGAV. Results of these studies functionally link ITGAV and SPP1 to Tr cell adhesion, an event central to implantation. With recent siRNA studies, we now know that SPP1 can directly adhere to ITGAV on both uterine epithelial cells (Kang *et al.* 2014) and on placental Tr cells (the present study), providing a strong support for the potential of SPP1 to act as a bridging ligand that tethers conceptus to uterus via transmembrane ITGAV-containing integrin receptors, as correctly predicted in 1997 (Aplin 1997).

Cultured porcine uterine LE and conceptus Tr cells were previously shown to express ITGAV, ITGB3 and

ITGB6 subunits that were bound to SPP1 (Erikson *et al.* 2009), and ITGAV and ITGB3 proteins were previously localized to the uterine LE and conceptus Tr in tissue sections through Day 20 of gestation in pigs (Bowen *et al.* 1996, Erikson *et al.* 2009); however, integrin expression beyond Day 20 had not been examined. In the present study, *in situ* hybridization confirmed the presence of mRNAs for ITGAV, ITGB3 and ITGB6 in both uterine LE and conceptus Tr with no change in the magnitude of expression over the course of pregnancy. Further, exogenous E2 or P4 did not affect ITGAV, ITGB3 and ITGB6 mRNA expression in the uteri of ovariectomized gilts. These results agree with a previous study that showed high expression of ITGAV and ITGB3 proteins at the apical surface of uterine LE that did not change over the peri-implantation period and were not regulated by sex steroids (Bowen *et al.* 1996). The present study is the first to report the expression of the ITGB6 subunit in the uteri or conceptuses of pigs. It is clear that in pigs, ITGAV, ITGB3 and ITGB6 mRNA expression is constitutive, and changes observed in protein expression are due to the redistribution of these integrin proteins within the tissues.

The present study is also the first to report protein expression for ITGAV, ITGB3 and SPP1 beyond Day 20 of

pig pregnancy. Immunofluorescence staining for ITGAV, ITGB3 and SPP1 proteins identified areas of intense apical punctate staining that appeared to aggregate into dense deposits in single cells or small groups of cells at the uterine-placental interface on Day 25 of pregnancy. Immunostaining detected ITGAV protein in both Tr and uterine LE cells, whereas ITGB3 immunostaining was limited to uterine LE. Similar results were previously reported at the uterine-placental interface of pigs on Days 20 of pregnancy, and it is key that Erikson and coworkers demonstrated changes in the distribution of α -actinin (ACTN; associated with adherens junctions in the same cells, which is indicative of the recruitment of ACTN at the apical surfaces of LE and Tr during assembly of Fas) (Erikson *et al.* 2009). However, in that previous study ITGAV and ATGB3 integrins appeared in aggregates, but SPP1 expression, although abundant, was uniform along the uterine-placental interface on Day 20 of gestation. In sharp contrast, SPP1 clearly forms intermittent punctate aggregates at the uterine-placental interface on Day 25 (Fig. 8). Integrin-mediated adhesion is focused within a primary mechanotransduction unit of dynamic structure and composition known as a FA whose size, composition, cell signaling activity and adhesion strength are force dependent (Hynes 1987, Vogel 2006). We hypothesize that aggregates of ITGAV, ITGB3 and SPP1 represent functionally distinct FAs that are formed in response to tensile or shear forces at the uterine-placental interface imposed during the attachment of the long filamentous Tr of porcine conceptuses to the uterine LE. The increased incorporation of SPP1 into these FAs between Days 20 and 25 may be due to the rapid elongation of the trophoblast that results in greater mechanical forces applied to connections between the placenta and uterus. The aggregation of SPP1 at this interface suggests that transmembrane ITGAV and ITGB3 are incorporated into FAs that span from the cytoplasmic plasma membrane surface (intracellular integrin subunit tails) to the intercellular space where they bind to SPP1.

Of particular interest in the present study is that ITGAV and ITGB3 immunostaining was no longer detectable, and SPP1 immunostaining became uniform (not punctate) at the uterine-placental interface on Days 50, 70 and 90 of porcine pregnancy. This temporal change in the distribution of integrin aggregates contrasts with what has been observed for sheep where FAs progressively increase as pregnancy progresses (Burghardt *et al.* 2009). Cells are variably exposed to mechanical forces, including tension, compression, shear and hydrostatic pressure, depending on their unique physiological environment (Nelson & Gleghorn 2012). Extracellular mechanical forces are sensed and translated into biochemical signals i.e., mechanosensation and mechanotransduction, within cells through FAs. FAs are complex structures that include ECM proteins bound to transmembrane integrins, and interaction of integrins with intracellular adaptor proteins, signaling

Figure 8 Immunofluorescence detection of SPP1 protein at the uterine-placental interface on Days 25, 35 and 60 of pregnancy. Large aggregates of SPP1 (OPN) protein were detected at the interface between luminal epithelial (LE) and conceptus trophoblast/chorionic epithelial (Tr) cells on Day 25, but were not detected on Days 35 or 60 of gestation. Width of fields for images obtained with a 16 $\times$ microscope objective is 540 μ m and 230 μ m for a 40 $\times$ objective magnification is 540 μ m and for the 40 $\times$ magnification is 230 μ m. Unlabeled arrows indicate the interface between LE and Tr.

complexes and actin-binding proteins to physically link to the cytoskeleton of the cell (Zaidel-Bar *et al.* 2007). As external force increases, FAs grow and mature, which leads to actin remodeling that produces intracellular forces necessary to balance the changing external force (Chen 2008, Zhang & Labouesse 2012). It has been hypothesized, for sheep, that the development of large FAs reflects an adaptation of the Tr/chorion to maintain a tight connection with uterine LE along regions of true epitheliochorial placentation in response to dynamic increases in tensile, compression and shear loads imposed by expanding fetal and placental mass and dynamic responses to changes in maternal posture,

locomotion and/or position of the fetus. A similar increase in FAs at the uterine-placental interface was predicted for pigs; however, this prediction did not take into account the dramatic increase in folding that is observed between the uterine endometrium and chorioallantois in pigs as compared to the interplacentomal regions of sheep, which do not exhibit folding of the uterine-placental interface. The attached endometrial-chorion epithelial bilayer develops microscopic folds, beginning about Day 35 of gestation, and these folds increase the area of contact between maternal and fetal capillaries to maximize maternal-to-fetal exchange of nutrients in pigs (Dantzer 1984). These folds may also serve to structurally redistribute and disperse forces generated by increasing tensile, compression and shear loads, removing the requirement for expansive FAs to maintain firm attachment between the uterus and placenta. As such, integrin function is not regulated by steroid hormones, but rather by changing mechanical forces applied to the uterine-placental interface.

In conclusion, results of the present study are the first to directly demonstrate that Tr cells engage ITGAV to adhere to SPP1 and suggest that attachment of the conceptus Tr to the uterine LE for implantation in pigs is mediated by ITGAV-containing integrin receptors that interact with SPP1 and perhaps other ECM ligands. These cell-ECM interactions respond to tension and shear forces generated by the act of tethering the elongating filamentous conceptuses to the uterine LE to form FAs. As implantation transitions into stable placentation, the endometrial epithelial-chorion bilayer develops folds to increase inter-digitation of uterine and placental tissues, thereby providing a 3-dimensional structure that disperses shear stress at the uterine-placental interface. Without these physical perturbations to drive FA assembly, there is an eventual loss of FAs at the uterine-placental interface.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-17-0043>.

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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