

Integrin αv is necessary for the attachment of trophoctoderm cells to osteopontin to mediate adhesion of the conceptus to the uterus during implantation in pigs

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Pigs experience a significant loss of conceptuses during the attachment phase of implantation. The molecules that facilitate uterine-conceptus interactions in pigs are not fully understood, and this gap in knowledge limits our ability to improve reproductive efficiency. Osteopontin (OPN, also known as Secreted Phosphoprotein 1 or SPP1) is an emerging candidate marker for uterine receptivity to implantation in pigs. Expression of OPN by the uterus increases markedly during the peri-implantation period of pigs, as well as in other mammalian species, including sheep, goats, rabbits, mice and humans (Johnson '03, Johnson '09). In pigs, OPN mRNA is initially induced by conceptus estrogens in discrete regions of the uterine luminal epithelium (ULE) juxtaposed to the conceptus just prior to implantation on Day 13. OPN mRNA expands to the entire ULE by Day 20 when there is firm adhesion of conceptus trophoctoderm to ULE (Garlow '02, White '05). OPN is a secreted extracellular matrix (ECM) protein that binds to cell surface integrins. Integrins are transmembrane glycoprotein receptors composed of non-covalently bound α and β subunits that promote cell-cell and cell-ECM adhesion, cause cytoskeletal reorganization to stabilize adhesion, and transduce signals through numerous signaling intermediates. OPN potentially binds multiple integrins including $\alpha v\beta 3$, $\alpha v\beta 1$, $\alpha v\beta 5$, $\alpha v\beta 6$, $\alpha 4\beta 1$, $\alpha 9\beta 1$ and $\alpha 8\beta 1$. Our central hypothesis is that OPN secreted by the ULE binds integrins expressed by both ULE and trophoctoderm to mediate implantation of the conceptus.

In a previous study, we tested whether αv , $\alpha 4$, $\alpha 5$, $\beta 1$, $\beta 3$, $\beta 5$, and $\beta 6$ integrin subunits expressed by porcine trophoctoderm cells (pTr2) and ULE cells bound OPN. OPN bound the $\alpha v\beta 6$ integrin heterodimer on pTr2 cells and $\alpha v\beta 3$ on ULE cells (Erikson '09). Further, OPN promoted dose- and integrin-dependent attachment of pTr2 and ULE cells, and stimulated haptotactic pTr2 cell migration, meaning that cells migrated directionally along a physical gradient of non-soluble OPN. Finally, OPN-coated microspheres revealed co-localization of the αv integrin subunit and talin to focal adhesions at the apical domain of pTr2 cells.

The objectives of the current studies were to: 1) determine mRNA expression and hormonal regulation of αv , $\beta 3$, and $\beta 6$ integrins in the uterus and placenta; 2) determine the temporal and spatial assembly of αv -, $\beta 3$ -, and $\beta 6$ -containing focal adhesions at the uterine-placental interface; and 3) determine whether integrin αv knockdown results in decreased attachment of pTr cells to OPN *in vitro*. In Experiment 1, gilts were hysterectomized on Day 9, 12 or 15 of the estrous cycle, or Day 9, 12, 15, 20, 25, 30, 35, 40, 50, 60 or 85 of pregnancy and uterine/placental tissues were subjected to *in situ* hybridization and immunofluorescence (IF) analyses to detect integrin subunit αv , $\beta 3$, and $\beta 6$ mRNA and αv and $\beta 3$ protein at the maternal-conceptus interface. In Experiment 2, cyclic gilts were injected daily (Days 11-14) with 17-beta-estradiol benzoate (i.m.) or vehicle, and hysterectomized on Day 15 of pseudopregnancy. In Experiment 3, gilts were ovariectomized on Day 12, injected daily with progesterone (i.m.) or vehicle for 28 days, and hysterectomized on Day 40. For Experiments 2 and 3, uteri were subjected to *in situ* hybridization analyses to determine

whether expression levels of αv , $\beta 3$ and $\beta 6$ mRNAs are regulated by estrogen and/or progesterone. In Experiment 4, custom smart pools of small interfering RNA (siRNA) targeted to GAPDH or pig αv integrin were delivered to pTr2 cells to silence GAPDH or αv gene expression respectively. Knock down of integrin subunit αv in siRNA treated pTr2 cells was confirmed by Western blotting and IF analyses. pTr2 cells were then seeded evenly in 96-well plates in which wells were coated with either BSA (negative control), bovine fibronectin (FN), OPN, or type I collagen (positive control). The cells were allowed to adhere for 1 h, stained with amido black and absorbance readings were taken at a wavelength of 595 nm. Treated cells were also placed on OPN coated cover slips and IF was performed to visualize the αv integrin subunit.

Interestingly *in situ* hybridization analysis demonstrated that αv , $\beta 3$, and $\beta 6$ mRNAs increased in ULE and trophoblast cells on Day 15 of pregnancy, peaked at Day 25, then decreased to background levels by Day 30 of gestation. No appreciable amounts of αv , $\beta 3$, or $\beta 6$ mRNAs were present in ULE of Day 15 pseudopregnant or Day 40 progesterone-treated gilts. IF staining detected large aggregates of αv protein at the apical surfaces of ULE and trophoblast cells, and $\beta 3$ aggregates on the ULE on Day 25 of pregnancy. Unexpectedly, these integrin aggregates, suggestive of focal adhesion assembly, were no longer detectable by Day 50. Finally, knocking down αv integrin decreased the adhesion of pTr2 cells to OPN and FN, but did not affect adhesion to type 1 collagen which does not bind αv (Figure 1A).

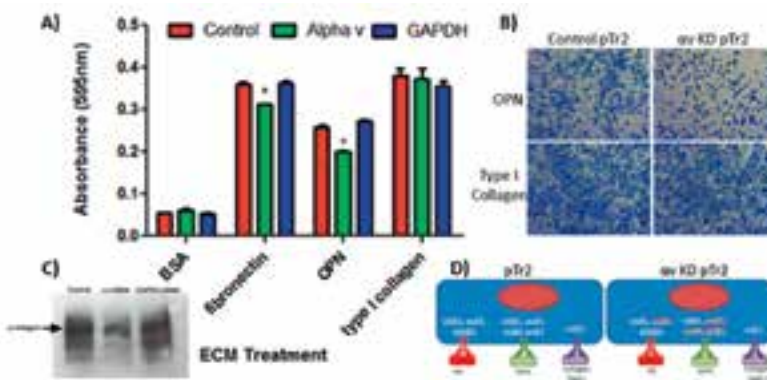


Fig. 1A. Adhesion of pTr2 cells to fibronectin (FN), OPN, or type I collagen. **B.** Photographs of pTr2 cells (control or αv knockdown (KD)) attached to OPN or type I collagen. **C.** Western blot showing significant KD of αv integrin subunit. **D.** Cartoon of integrin expression in wild-type pTr2 cells (left panel) and pTr2 cells treated with siRNA directed to the αv integrin subunit (right panel).

Novel results from the current study in pigs are: 1) large focal adhesions form to adhere ULE and placental trophoblast only during the peri-implantation period; 2) integrin mRNA increases in LE and trophoblast during the peri-implantation period, and the up-regulation is not the result of estrogen or progesterone, suggesting that focal adhesion formation may lead to outside-in integrin signaling to increase integrin transcription in a positive feedback loop; and 3) αv integrin is necessary for the attachment of trophoblast cells to OPN *in vitro*, suggesting a similar during implantation in pigs.

References

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