Association of foetal size and sex with porcine foeto-maternal interface integrin expression

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Abstract

Integrins regulate adhesion at the foeto-maternal interface by interacting with secreted phosphoprotein 1 (SPP1) and fibronectin (FN). It is hypothesised that impaired foetal growth of 'runt' piglets is linked to altered integrin signalling at the foeto-maternal interface. Placental and endometrial samples associated with the lightest and closest to mean litter weight (CTMLW) (gestational day (GD) 18, 30, 45, 60 and 90), of both sex (GD30, 45, 60 and 90) ($n=5-8$ litters/GD), Large White $\times$ Landrace conceptuses or foetuses were obtained. The mRNA expression of the integrin subunits (ITG) *ITGA2*, *ITGAV*, *ITGB1*, *ITGB3*, *ITGB5*, *ITGB6*, *ITGB8*, *SPP1* and *FN* was quantified by qPCR. Temporal changes in mRNA expression were observed, with different profiles in the two tissues. Endometrial *ITGB1* ($P \leq 0.05$, GD45) and *SPP1* ($P \leq 0.05$, all GD combined and GD60) expression was decreased in samples supplying the lightest compared to the CTMLW foetuses. Placentas supplying female foetuses had decreased expression of *ITGB6* (GD45, $P \leq 0.05$) and *FN* (GD90, $P \leq 0.05$) compared to those supplying male foetuses. Endometrial samples supplying females had increased *ITGB3* ($P \leq 0.05$, GD60) and *FN* ($P \leq 0.05$, GD30) expression and decreased *SPP1* ($P \leq 0.05$, GD60) expression compared to male foetuses. Correlations between mean within-gilt mRNA expression and percentage prenatal survival, number of live foetuses or conceptuses and percentage male foetuses were observed. This study has highlighted novel and dynamic associations between foetal size, sex and integrin subunit mRNA expression at the porcine foeto-maternal interface. Further studies should be performed to improve the understanding of the mechanisms behind these novel findings.

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Introduction

Low birthweight infants have an increased risk of mortality and morbidity and can be classified as small for gestational age (SGA) or intra-uterine growth restricted (IUGR). The pig has a high incidence of naturally occurring low birthweight piglets, with many litters having a growth-restricted or 'runt' piglet that can weigh less than half of the weight of their largest littermates (Widdowson 1971, Ashworth *et al.* 2001, Wu *et al.* 2010). Considering the high prevalence of low birth weight piglets, it is essential to improve the understanding of the mechanisms governing foetal growth. It is hypothesised that inadequate conceptus attachment to the endometrium contributes to the large variation in piglet weight and high prevalence of low birthweight piglets.

Integrins (ITGs) are glycoprotein transmembrane receptors which exist as heterodimers composed of non-covalently linked alpha (ITGA) and beta (ITGB) subunits (Giancotti & Ruoslahti 1999). Integrins play a central role in cell adhesion and have been shown to be critical in the

formation of focal adhesions, cell migration, proliferation and the development of the actin cytoskeleton (Irving & Lala 1995, Schwartz & Assoian 2001, Gallant *et al.* 2005, Delon & Brown 2007). Specific integrin heterodimers can bind to peptides containing an Arg-Gly-Asp (RGD) region, such as secreted phosphoprotein 1 (SPP1, also known as osteopontin (OPN)) and fibronectin (FN). The process of implantation requires significant remodelling of the extracellular matrix at the interface between the conceptus and the mother (Carson *et al.* 2000, White *et al.* 2006), which is believed to be partially regulated by integrin signalling. Both SPP1 (Feinberg *et al.* 1991, Denker 1993, Sutherland *et al.* 1993, Aplin *et al.* 1994, Lessey *et al.* 1994, Ruck *et al.* 1994, Campbell *et al.* 1995, Fassler & Meyer 1995, Schultz & Armant 1995, Stephens *et al.* 1995, Yoshimura *et al.* 1995, Bowen *et al.* 1996, Shiokawa *et al.* 1996, 1999, Fazleabas *et al.* 1997, Schultz *et al.* 1997, Yelich *et al.* 1997, Guillomot 1999, Hodivala-Dilke *et al.* 1999, Johnson *et al.* 1999, 2001, Kimmins & MacLaren 1999, Illera *et al.* 2000, 2003, Bloor *et al.* 2002, Burghardt *et al.* 2002, 2009, Kao

et al. 2002, Qin *et al.* 2003, Rashev *et al.* 2005, White *et al.* 2005, Zheng *et al.* 2006, Lin *et al.* 2007, Erikson *et al.* 2009a, Germeyer *et al.* 2014, Vélez *et al.* 2015, 2017, Frank *et al.* 2017) and FN (Feinberg *et al.* 1991, Schultz & Armant 1995, Bowen *et al.* 1996, Qin *et al.* 2003, Rashev *et al.* 2005, Imakawa *et al.* 2006, Zheng *et al.* 2006, Vélez *et al.* 2015, 2017) have been reported to play a role in early gestation in multiple species by regulating integrin signalling. In the pig, trophoblast and uterine luminal epithelial cells have been shown to express *ITGAV*, *ITGA2*, *ITGA5*, *ITGB1*, *ITGB3* and *ITGB6* (Bowen *et al.* 1996, Erikson *et al.* 2009a, Frank *et al.* 2017). It has been suggested that $ITG\alpha 5\beta 1$, $ITG\alpha V\beta 3$, $ITG\alpha V\beta 6$ and $ITG\alpha V\beta 1$ receptors may be able to bind to SPP1, and FN may bind to $ITG\alpha 5\beta 1$, $ITG\alpha V\beta 3$ or $ITG\alpha II\beta 3$ (Humphries *et al.* 2006). Further, porcine trophoblast cells have been shown to utilise integrin heterodimers containing *ITGAV* receptors to bind to SPP1 during implantation (Frank *et al.* 2017).

In addition to a central role in the regulation of implantation and early pregnancy in the pig (Bowen *et al.* 1996, Rashev *et al.* 2005, White *et al.* 2005, Erikson *et al.* 2009a, Vélez *et al.* 2015, 2017), integrins and their ligands are expressed at the porcine foeto-maternal interface at multiple gestational days (GD) (Garlow *et al.* 2002, White *et al.* 2005, Hernández *et al.* 2013, Vélez *et al.* 2015, 2017, Frank *et al.* 2017, Steinhäuser *et al.* 2017). Considering the non-invasive nature of the porcine placenta (Montiel *et al.* 2013), and the substantial remodelling that must occur to ensure adequate nutrient transfer to the foetus (Vallet & Freking 2007), it is unsurprising that adhesive processes must be present throughout gestation to maintain a successful pregnancy.

It is hypothesised that the mRNA expression of integrin subunits, FN and *SPP1*, will be decreased in placental and endometrial samples associated with the lightest conceptuses or foetuses compared to their normal-sized littermates throughout gestation. To test this hypothesis, samples were compared within litter at five GDs of interest.

It has previously been demonstrated that significant variation in foetal size can be observed at GD30 (Pettigrew *et al.* 1986, Wise *et al.* 1997, Finch *et al.* 2002, Foxcroft & Town 2004, Foxcroft *et al.* 2006), which is thought to be reflective of the postnatal within-litter variation in the piglet size observed. Therefore, placental and endometrial samples from early gestation were utilised as part of this study. During pregnancy, temporal changes in the rate of foetal and placental growth can be observed (Marrable 1971). After the initially fast period of placental growth, the growth rate of the porcine placenta plateaus in mid-gestation, whereby instead of continuing to increase in weight, the structure of the placenta undergoes extensive remodelling to increase the surface area available for nutrient exchange with the developing foetus (Vallet

& Freking 2007). Whilst the placental growth rate is decreased, the foetus is undergoing rapid growth, placing large demands upon the placenta. This study has utilised placental and endometrial samples from GD18, 30, 45, 60 and 90, which were selected to allow investigation of these tissues throughout gestation due to the dynamic relationship between foetal and placental growth at the foeto-maternal interface.

Recent literature has suggested that sexual dimorphism in placental development exists in humans (Rosenfeld 2015, Kalisch-Smith *et al.* 2017), which may translate to the observed sexual dimorphism in phenotype in response to adverse conditions during pregnancy. In the pig, it has been suggested that males are at a disadvantage compared to their female littermates post-natally (Baxter *et al.* 2012). Further, recent investigations in our laboratory have reported sexual dimorphism in both placental and endometrial vascularity in the pig (Stenhouse 2018, Stenhouse *et al.* 2018a). Considering this, it is hypothesised that decreased expression of the integrin receptors and their ligands will be observed in placental and endometrial samples supplying male foetuses compared to their female littermates.

The number of live born piglets per year is an economically important factor for the pig industry (Koketsu *et al.* 2017). Further, the sex ratio of the litter is known to influence reproductive success (Lamberson *et al.* 1988, Drickamer *et al.* 1997, 1999, Huhn *et al.* 2002, Górecki 2003). Considering this, and the proposed role of integrin signalling at the foeto-maternal interface, the relationship between integrin mRNA expression and percentage prenatal survival, number of live foetuses and the percentage of male foetuses in the litter was investigated.

Materials and methods

All procedures were performed with approval from The Roslin Institute (University of Edinburgh) Animal Welfare and Ethical Review Board and in accordance with the U.K. Animals (Scientific Procedures) Act, 1986.

Experimental animals and sample collection

Large White $\times$ Landrace gilts (age 11–14 months; $n=31$) were observed daily for signs of oestrus and were housed in groups of 6–8 animals per pen. Oestrous cyclicity and ovarian function were controlled in accordance with routine normal practice at The Roslin Institute Large Animal Unit. In a subset of gilts (distribution between the GD investigated indicated in Supplementary Table 1, see section on [supplementary data](#) given at the end of this article) oestrus was synchronised by daily feeding of 20 mg Altrenogest (Regumate, Hoechst Roussel Vet Ltd.) for 18 days followed by injection of pregnant mare serum gonadotrophin (PMSG; Intervet UK Ltd) and human chorionic gonadotrophin (hCG; Intervet UK Ltd) (Stenhouse *et al.* 2018a). All gilts were inseminated twice daily for the

duration of oestrus with semen from one of four Large White boars. The boars used were equally distributed throughout the GD to minimise any effect of sire. The first day of insemination was assigned as GD0 and samples were obtained at GD18, 30, 45, 60 and 90. Gilts were killed at the GD of interest with sodium pentobarbitone 20% w/v (Henry Schein Animal Health) at a dose of 0.4 mL/kg by intravenous injection via a cannula inserted in the ear vein. Following confirmation of death, mid-ventral incision revealed the reproductive tract. The tract was lifted from the body cavity and placed in a dissecting tray. Both uterine horns were dissected, from the ovary towards the cervix. The uterine lumen was occluded between each foeto-placental unit by tying with string to ensure that the tissue associated with particular conceptuses or foetuses could be identified later.

At GD18, the uterine tract was rinsed with saline and a string was used to tie the end of the right and left uterine horns at the bifurcation. The uterine horns were cut between the two pieces of string and each uterine horn was placed in a floatation device. The device contained a solution to preserve the integrity of the RNA (700g ammonium sulphate (SLS) was dissolved in 935 mL of RNase-free water with heat and stirring). Once dissolved, 25 mL of 1 M sodium citrate (Fisher Scientific) and 40 mL of 0.5 M EDTA were added. The solution was adjusted to pH 5.2 using concentrated sulphuric acid and stored at room temperature until required. Using dissection scissors, the uterine horn was opened along the mesometrial side, and the conceptuses floated in the solution. Individual conceptuses were removed from the floatation device with forceps and weighed in a cryovial (Starlab). The uterine lumen was occluded between each conceptus to ensure that endometrial samples associated with particular conceptuses could be identified. The lightest and CTMLW conceptus was identified based on weight and snap-frozen in liquid nitrogen and stored at -80°C for RNA extraction.

On the remaining GD investigated, foetuses were identified as 'live' or 'dead' based on their morphology at the time of dissection and were weighed. At GD45, 60 and 90, foetal sex was determined morphologically. DNA was isolated from the GD30 foetuses using the DNeasy Blood and Tissue DNA extraction kit (Qiagen), and PCR was performed for the sex-determining region Y (Sry) – a region of the Y chromosome – as previously described (Stenhouse *et al.* 2018b) to determine the sex of the foetuses. The lightest and CTMLW foetuses (GD30), of both sex (GD45, 60 and 90), were identified based on foetal weight. From the anti-mesometrial side, placental and endometrial samples were taken from each foeto-placental unit of interest, snap-frozen in liquid nitrogen and stored at -80°C for RNA extraction.

Analysis of mRNA expression by qPCR

Total RNA extraction and cDNA synthesis

RNA was extracted from 20 to 50 μg of snap-frozen placental and endometrial samples as previously described (Stenhouse *et al.* 2018a). The RNA was quantified, and the quality was assessed spectrophotometrically using a Nanodrop ND-1000

(Labtech International Ltd.) and electrophoretically using a TapeStation 2200 (Agilent Technologies). The mean A260/A280 and RNA Integrity Number Equivalent (RINe) for samples within each GD are detailed in Supplementary Table 2. Extracted RNA was stored at -80°C until required. If the RINe value obtained remained lower than the ranges detailed in Supplementary Table 2, the sample was excluded from the analyses.

cDNA was prepared from 0.3 μg of RNA with SuperScript III reverse transcriptase (Life Technologies) following the manufacturer's instructions. Each reaction contained 250 ng random primers (Promega) and 40 units RNaseIn (Promega). Negative controls without reverse transcriptase were included to check for genomic contamination. Reverse transcription was performed in duplicate for each sample and pooled, and the cDNA was stored at -20°C until required.

Relative expression of candidate genes

Quantitative PCR was performed on a Stratagene MX3000 instrument using Platinum SYBR Green SuperMixUTG (Life Technologies) using cDNA from placental (GD30, 45, 60 and 90; $n=6, 6, 6$ and 8 litters respectively) and endometrial (GD18, 30, 45, 60 and 90; $n=5, 5, 6, 6$ and 6 litters respectively) samples. The samples were associated with the lightest and CTMLW conceptuses or foetuses at GD18 and 30, and the lightest and CTMLW foetuses of both sex at GD45, 60 and 90. The final concentrations of magnesium, ROX reference dye and each primer were 3 mM, 50 nM and 400 nM respectively in a 25 μL reaction volume. All qPCRs were carried out at an annealing temperature of 60°C and dissociation curves consisting of single peaks were generated. The mRNA expression of *ITGA2*, *ITGAV*, *ITGB1*, *ITGB3*, *ITGB5*, *ITGB6*, *ITGB8*, *SPP1* and *FN* was quantified in both tissues (King *et al.* 2011, Hernández *et al.* 2013, Frank *et al.* 2017). Appropriate reference genes were identified by analysis of 11 candidate reference genes (Erkens *et al.* 2006, Nygard *et al.* 2007) using geNORM V3.5 (Ghent University Hospital, Centre for Medical Genetics). The reference genes *TBP1* (TATA box-binding protein) and *HPRT1* (hypoxanthine phosphoribosyltransferase 1) were utilised to normalise placental mRNA expression, and endometrial mRNA expression was normalised using the reference genes *TBP1*, *YWHAZ* (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein and zeta polypeptide) and *TOP2B* (Topoisomerase II beta). The primer sequences for all genes are detailed in Supplementary Table 3.

Serial dilutions of pooled cDNA ranging from 1:5 to 1:640 in nuclease-free water were used as standards. Sample cDNA was diluted 1:25 and 5 μL of diluted sample, standard or control were added per well. Each plate contained duplicate wells of a no template control, standards, sample cDNA and reverse transcriptase blanks. Data were analysed using qbase+ software V3.0 (Biogazelle). A target- and run-specific strategy was employed and the results, normalised to the two reference genes, were scaled to the minimum sample. The mean slope, intercept, PCR efficiency and R^2 values are detailed in Supplementary Table 4.

Statistical analysis

All statistical analyses were performed using Minitab 17 or GenStat 13.1 (VSN International Ltd.). The normalised mean value for each placental and endometrial sample was taken and the normality of the distribution of the data was assessed by an Anderson-Darling test. If a P value of ≤ 0.05 was obtained, then the data were not considered to have a normal distribution. Outliers were tested for using a Grubbs outlier test and were excluded systematically, with normality within each group being reassessed following each exclusion. Log₁₀ transformations were carried out where appropriate to improve the normality of the distribution of the data. The effect of foetal size was assessed by comparing values for the true lightest and true CTMLW at GD18, 30, 45, 60 and 90. Effects of foetal sex were assessed by comparing values from placental and endometrial samples supplying foetuses of both sex at GD30, 45, 60 and 90. Where data had a normal distribution, ANOVA for GD, foetal size or sex was performed, with a block for gilt to account for the common maternal environment. A *post hoc* Tukey test was performed where appropriate. Where data did not have a normal distribution, Kruskal-Wallis and Mann Whitney tests were performed. Within gilt, the mean expression for each gene of interest was calculated within tissue. Pearson's correlations were performed within GD to determine the association between the mean gene expression within gilt and the percentage of males in the litter (sex ratio), percentage prenatal survival (calculated by dividing the number of live foetuses by the number of *corpora lutea*, multiplied by 100) and the number of live foetuses. In all experiments, results were considered significant when $P \leq 0.05$, tending towards significant when P was >0.05 and <0.1 and not significant when $P \geq 0.1$.

Results

Temporal changes in integrin mRNA expression

No significant day effect was observed in the placental expression of *ITGB5* or *SPP1* (Fig. 1E and I). An overall day effect was observed in the placental expression of *ITGA2* ($P \leq 0.05$; Fig. 1A), with GD30 placentas having decreased expression compared to the other GD investigated. Placental *ITGAV* expression was influenced by GD ($P \leq 0.05$; Fig. 1B), with decreased expression observed at GD60 compared to GD30 and GD45. An overall GD effect was observed in the placental expression of *ITGB1* ($P \leq 0.001$; Fig. 1C), with increased expression observed at GD45 and GD90 compared to GD30 and GD60. Placental *ITGB3* expression was decreased at GD60 and GD90 compared to GD45 ($P \leq 0.05$; Fig. 1D). Temporal changes in the placental expression of *ITGB6* were observed ($P \leq 0.01$; Fig. 1F), with low placental expression at GD30, which significantly increased at GD45. Placental *ITGB6* expression then significantly increased to its peak expression level at GD90. Placental *ITGB8* expression increased with advancing gestation ($P \leq 0.01$; Fig. 1G), with a significant increase observed between GD30 and GD45. Placental *FN* expression varied throughout gestation ($P \leq 0.01$; Fig. 1H).

An overall GD effect was observed in *ITGA2* endometrial expression ($P \leq 0.05$; Fig. 2A). The endometrial expression of *ITGAV* ($P \leq 0.001$; Fig. 2B) and *ITGB3* ($P \leq 0.01$; Fig. 2D) were decreased at GD30 compared to the other GD investigated. During

Figure 1 Integrin mRNA expression in placental tissues on days 30, 45, 60 and 90 of pregnancy. mRNA expression of *ITGA2* (A), *ITGAV* (B), *ITGB1* (C), *ITGB3* (D), *ITGB5* (E), *ITGB6* (F), *ITGB8* (G), *FN* (H) and *SPP1* (I) in placental samples at GD30, 45, 60 and 90. Error bars represent s.e.m. $n = 9-23$ foetuses/GD.

Figure 2 Integrin mRNA expression in endometrial tissues on days 18, 30, 45, 60 and 90 of pregnancy. mRNA expression of *ITGA2* (A), *ITGAV* (B), *ITGB1* (C), *ITGB3* (D), *ITGB5* (E), *ITGB6* (F), *ITGB8* (G), *FN* (H) and *SPP1* (I) in endometrial samples at GD18, 30, 45, 60 and 90. Error bars represent s.e.m. $n=9-30$ fetuses/GD.

gestation, *ITGB1* ($P \leq 0.01$; Fig. 2C) and *ITGB8* ($P \leq 0.001$; Fig. 2G) endometrial expression fluctuated. Endometrial *ITGB5* expression also fluctuated throughout gestation ($P \leq 0.001$; Fig. 2E), with decreased expression observed at GD30, GD60 and GD90 compared to GD18 and GD45. *ITGB6* endometrial expression was increased in mid and late gestation compared to early gestation, with a notable increase in expression observed between GD30 and GD45 ($P \leq 0.001$; Fig. 2F). Endometrial

SPP1 expression increased with advancing gestation ($P \leq 0.001$; Fig. 2I).

Associations between foetal size and integrin mRNA expression

No statistically significant associations between foetal size and placental expression of *ITGAV* (Fig. 3B), *ITGB1* (Fig. 3C), *ITGB3* (Fig. 3D), *ITGB5* (Fig. 3E),

Figure 3 Integrin mRNA expression in placental tissues associated with the lightest and CTMLW conceptuses and fetuses on days 30, 45, 60 and 90 of pregnancy. mRNA expression of *ITGA2* (A), *ITGAV* (B), *ITGB1* (C), *ITGB3* (D), *ITGB5* (E), *ITGB6* (F), *ITGB8* (G), *FN* (H) and *SPP1* (I) in placental samples associated with the lightest and CTMLW fetuses at GD30, 45, 60 and 90. Error bars represent s.e.m. $n=4-7$ fetuses/GD.

ITGB6 (Fig. 3F), *ITGB8* (Fig. 3G), *FN* (Fig. 3H) or *SPP1* (Fig. 3I) were observed. At GD45 ($P=0.07$; Fig. 3A), placental samples supplying the lightest foetuses tended to have increased *ITGA2* expression compared to those supplying the CTMLW foetuses. The direction of this difference switched at GD90 ($P=0.09$; Fig. 3A), with placental samples supplying the lightest foetuses having decreased *ITGA2* expression compared to those supplying the CTMLW foetuses.

No statistically significant associations between conceptus or foetal size and endometrial expression of *ITGA2* (Fig. 4A), *ITGAV* (Fig. 4B), *ITGB3* (Fig. 4D), *ITGB5* (Fig. 4E), *ITGB6* (Fig. 4F) or *FN* (Fig. 4H) were observed. Endometrial *ITGB1* expression was decreased in samples supplying the lightest foetuses compared to those supplying the CTMLW foetuses at GD45 ($P\leq 0.05$; Fig. 4C). At GD30, a trend towards endometrial samples supplying the lightest foetuses having increased *ITGB5* expression compared to samples supplying the CTMLW foetuses was observed ($P=0.07$; Fig. 4E). Further, endometrial samples associated with the lightest conceptuses tended ($P=0.09$; Fig. 4G) to have increased *ITGB8* expression compared to the CTMLW conceptuses at GD18. An overall size effect in *SPP1* expression was observed ($P\leq 0.05$; Fig. 4I), with a statistically significant decrease in expression observed in endometrial samples supplying the lightest foetuses compared to the CTMLW foetuses at GD60 ($P\leq 0.05$).

Associations between foetal sex and integrin mRNA expression

No statistically significant associations between foetal sex and placental expression of *ITGA2* (Fig. 5A), *ITGAV* (Fig. 5B), *ITGB1* (Fig. 5C), *ITGB3* (Fig. 5D), *ITGB5* (Fig. 5E), *ITGB8* (Fig. 5G) or *SPP1* (Fig. 5I) were observed. Significant associations between foetal sex and *ITGB6* (GD45 $P\leq 0.05$; Fig. 5F) and *FN* (GD90 $P\leq 0.05$; Fig. 5H) expression were observed, with placentas associated with female foetuses having decreased expression compared to those associated with male foetuses.

No statistically significant associations between foetal sex and endometrial expression of *ITGA2* (Fig. 6A), *ITGAV* (Fig. 6B), *ITGB1* (Fig. 6C), *ITGB5* (Fig. 6E), *ITGB6* (Fig. 6F) or *ITGB8* (Fig. 6G) were observed. Endometrial samples supplying females at GD60 had increased *ITGB3* expression compared to those supplying male foetuses ($P\leq 0.05$; Fig. 6D). At GD30, *FN* expression was increased in endometrial samples supplying females compared to those supplying male foetuses ($P\leq 0.05$; Fig. 6H). *SPP1* endometrial expression was decreased in samples supplying female foetuses compared to their male littermates at GD60 ($P\leq 0.05$; Fig. 6I).

Associations between mean gilt placental and endometrial integrin expression and litter characteristics

At GD30, an inverse correlation between mean placental *ITGB1* expression and percentage of males in the litter

Figure 4 Integrin mRNA expression in endometrial tissues associated with the lightest and CTMLW conceptuses and foetuses on days 18, 30, 45, 60 and 90 of pregnancy. mRNA expression of *ITGA2* (A), *ITGAV* (B), *ITGB1* (C), *ITGB3* (D), *ITGB5* (E), *ITGB6* (F), *ITGB8* (G), *FN* (H) and *SPP1* (I) in endometrial samples associated with the lightest and CTMLW conceptuses and foetuses at GD18, 30, 45, 60 and 90. Error bars represent S.E.M. * $P\leq 0.05$. $n=3-8$ foetuses/GD.

Figure 5 Integrin mRNA expression in placental tissues associated with male and female fetuses on days 30, 45, 60 and 90 of pregnancy. mRNA expression of *ITGA2* (A), *ITGAV* (B), *ITGB1* (C), *ITGB3* (D), *ITGB5* (E), *ITGB6* (F), *ITGB8* (G), *FN* (H) and *SPP1* (I) in placental samples associated with male and female fetuses at GD30, 45, 60 and 90. Error bars represent S.E.M. * $P \leq 0.05$. $n = 4-12$ fetuses/GD.

was observed ($P \leq 0.01$; Table 1). Placental *ITGAV* (GD60 and GD90 $P \leq 0.05$), *ITGB5* (GD90 $P \leq 0.001$), *ITGB6* (GD90 $P \leq 0.01$), *ITGB8* (GD90 $P \leq 0.01$) and *FN* (GD90 $P \leq 0.001$) expression were positively correlated with the number of live fetuses (Table 1).

A positive correlation between both mean endometrial *ITGB6* (GD45 $P \leq 0.05$) and *FN* (GD90 $P \leq 0.05$) expression and the percentage of males was observed (Table 1). Both endometrial *SPP1* (GD18 $P \leq 0.05$) and *ITGAV* (GD45 $P \leq 0.01$) expression were positively

correlated with percentage prenatal survival (Table 1). Endometrial *ITGB5* (GD18 $P \leq 0.01$) and *ITGB8* (GD30 $P \leq 0.05$) were inversely correlated with the number of live conceptuses or fetuses (Table 1).

Discussion

Integrin signalling has been heavily implicated in the establishment and maintenance of pregnancy by regulating adhesion at the foeto-maternal interface.

Figure 6 Integrin mRNA expression in endometrial tissues associated with male and female fetuses on days 30, 45, 60 and 90 of pregnancy. mRNA expression of *ITGA2* (A), *ITGAV* (B), *ITGB1* (C), *ITGB3* (D), *ITGB5* (E), *ITGB6* (F), *ITGB8* (G), *FN* (H) and *SPP1* (I) in endometrial samples associated with male and female fetuses at GD30, 45, 60 and 90. Error bars represent S.E.M. * $P \leq 0.05$. $n = 3-16$ fetuses/GD.

Table 1 Significant correlations between integrin mRNA expression and litter characteristics.

Gestational day	Tissue	Gene	Variable	RSq (%)	P value	Number XY pairs	Positive/Negative
30	Placenta	<i>ITGB1</i>	Percentage male foetuses	85.9	≤0.01	6	Negative
60	Placenta	<i>ITGAV</i>	Number of live foetuses	64.6	≤0.05	6	Positive
90	Placenta	<i>ITGAV</i>	Number of live foetuses	70.3	≤0.05	7	Positive
90	Placenta	<i>ITGB5</i>	Number of live foetuses	90.0	≤0.001	7	Positive
90	Placenta	<i>ITGB6</i>	Number of live foetuses	74.0	≤0.01	7	Positive
90	Placenta	<i>ITGB8</i>	Number of live foetuses	76.2	≤0.01	7	Positive
90	Placenta	<i>FN</i>	Number of live foetuses	89.3	≤0.001	7	Positive
18	Endometrium	<i>SPP1</i>	Percentage prenatal survival	79.7	≤0.05	5	Positive
18	Endometrium	<i>ITGB5</i>	Number of live conceptuses	94.7	≤0.01	5	Negative
30	Endometrium	<i>ITGB8</i>	Number of live foetuses	91.1	≤0.05	4	Negative
45	Endometrium	<i>ITGB6</i>	Percentage male foetuses	77.9	≤0.05	6	Positive
45	Endometrium	<i>ITGAV</i>	Percentage prenatal survival	84.3	≤0.01	6	Positive
90	Endometrium	<i>FN</i>	Percentage male foetuses	73.1	≤0.05	6	Positive

This study demonstrated an association between mRNA expression of integrins and their ligands, and foetal size at the porcine foeto-maternal interface. There were also novel relationships between foetal sex and both endometrial and placental mRNA expression. Intriguingly, differences in gene expression observed in placental and endometrial tissue appear to occur independently of one another.

This study identified novel associations between foetal size and the endometrial expression of *ITGB1* and *SPP1*. In humans, it has been shown that extravillous trophoblast cells in term placentas which supplied IUGR infants had decreased expression of *ITGα2β1*, *ITGα3β1* and *ITGα5β1* integrin receptors compared to those which supplied normally grown infants (Zygmunt *et al.* 1997). In the current study, endometrial samples supplying the lightest foetuses had decreased *ITGB1* expression at GD45 compared to those supplying their normal-sized littermates, reinforcing the suggestion *ITGB1* and its ligands may have a role in the regulation of foetal growth in the pig. Further, the *ITGα5β1* receptor has been suggested to bind both *FN* and *SPP1* at the foeto-maternal interface in the pig (Frank *et al.* 2017). Whilst no decrease in the expression of *SPP1* or *FN* were observed in the endometrial samples supplying the lightest foetuses compared to their normal-sized littermates at GD45, decreased endometrial *SPP1* expression was observed at GD60.

Emerging evidence in humans suggests that sexual dimorphism in placental development is responsible for sexual differences to disease susceptibility post-natally (Rosenfeld 2015, Kalisch-Smith *et al.* 2017). It has been proposed that male new-born piglets have a survival disadvantage compared to their female littermates (Baxter *et al.* 2012). However, whether this difference arises prenatally due to sexual dimorphism in placental development has not been determined in the pig. Currently, the suggestion of sexual dimorphism in placental or endometrial integrin expression in any species has not been explored. However, in a recent RNA sequencing experiment using human

placentas from 10.5 to 13.5 weeks, it was identified that placentas supplying female foetuses had increased *ITGB8* expression compared to those supplying males (Gonzalez *et al.* 2018).

Endometrial *FN* and *ITGB3* expression were increased in samples associated with female foetuses compared with their male littermates at GD30 and 60 respectively. The differential expression of *FN* in the endometrium but not the placenta at this early stage of gestation may indicate the presence of differential signalling between the conceptus and the endometrium in early gestation, although the mechanisms behind this require further investigation.

An inverse relationship between the percentage of males in the litter and placental *ITGB1* (GD30) expression and positive correlations between both endometrial *ITGB6* (GD45) and *FN* (GD90) expression and the percentage of males in the litter were observed. Intriguingly, whilst these results do not mirror the sex differences observed within tissue within GD, placental *ITGB6* and *FN* were demonstrated to be associated with foetal sex at GD45 and GD90 respectively. Interestingly, endometrial *ITGB5* and *ITGB8* expression were inversely correlated with the number of live conceptuses or foetuses in early gestation, whereas in late gestation placental *ITGAV*, *ITGB5*, *ITGB6*, *ITGB8* and *FN* expression were positively correlated with the number of live foetuses. The width of the folded bilayer at the foeto-maternal interface is known to increase significantly from GD65 to GD105 to increase the available surface area for exchange, allowing adequate nutrient transfer to meet the demands of the exponentially growing foetus (Vallet & Freking 2007). Further, there is some evidence to suggest that as litter size increases, uterine capacity becomes a limiting factor (Ford *et al.* 2002, Vallet *et al.* 2014), resulting in smaller placentas. It is proposed that increased remodelling of the interface must occur to attempt to improve placental efficiency and maintain adequate nutrient transfer to the developing foetus. The observed increase in gene expression in late gestation would suggest increased cell adhesion during this

period of remodelling. These novel correlations between integrin mRNA expression and number of live foetuses, percentage prenatal survival and percentage of male foetuses in the litter which, considering the importance of these characteristics post-natally, may be of interest to investigate further.

Importantly, this study has demonstrated that endometrial *SPP1* expression was associated with both foetal size and sex, suggesting the actions of *SPP1* at the foeto-maternal interface are associated with foetal development. Binding of *SPP1* to the ITGAVB3 receptor has been demonstrated to activate the Akt (Protein Kinase B)/eNOS (endothelial nitric oxide synthase) signalling pathway, leading to increased proliferation, migration and tube formation of endothelial cells *in vitro* (Dai *et al.* 2009, Wang *et al.* 2011). Dunlap *et al.* (2008) demonstrated in the sheep a relationship between uterine von Willebrand Factor (vWF) staining and *SPP1*, and that uterine arterial endothelial cells produce *SPP1* during angiogenesis *in vitro*. Analysis of GD9, 12 and 15 porcine endometrial samples identified the presence of a 32kDa fragment of *SPP1*, which is known to bind to the cell surface of endothelial cells (Erikson *et al.* 2009b, Bayless *et al.* 2010). In these studies, *in vitro* experiments using blood samples from new-born piglets demonstrated that *SPP1* has a positive effect on the migration and adhesion of endothelial cells, suggesting a potential role in regulating placental and endometrial angiogenesis. At the pig foeto-maternal interface, there are two key stages of gestation when angiogenesis occurs. The first is at ~GD13-18 (Keys *et al.* 1986), which corresponds to the period of conceptus attachment, and the second wave of angiogenesis occurs at approximately GD50 (Vonnahme *et al.* 2001), which corresponds to the period when placental growth is beginning to plateau and the foetus is about to undergo exponential growth. An additional study utilising the same samples used in the present study revealed striking associations between foetal size and sex and angiogenesis at the foeto-maternal interface, which were dependent on the GD investigated (Stenhouse 2018a,b). Therefore, considering the role of *SPP1* and ITGAVB3 during implantation (Erikson *et al.* 2009a, Frank *et al.* 2017) and the results of the current study, it could be hypothesised that *SPP1* has an additional role in inducing altered angiogenesis in foetuses of different size and sex which should be investigated further.

Conclusion

A comprehensive temporal analysis of the mRNA expression of key integrin subunits, *FN* and *SPP1* throughout gestation in both placental and endometrial samples was performed, identifying previously undescribed temporal changes in integrin expression. Endometrial expression of *ITGB1* and *SPP1* was decreased in samples associated with the lightest

foetuses compared to samples supplying their normally grown littermates at GD45 and 60 respectively. Overall, this demonstrated that foetal size is related to integrin signalling at the foeto-maternal interface but surprisingly this appears to occur in a temporal manner. Novel associations between foetal sex and the expression of *ITGB3*, *ITGB6*, *SPP1* and *FN* at the foeto-maternal interface were also observed. The results presented in this study are intriguing and the mechanisms behind these differences warrant further investigation, especially during preimplantation development and early pregnancy.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0520>.

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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References

- Aplin JD, Seif MW, Graham RA, Hey NA, Behzad F & Campbell S 1994 The endometrial cell surface and implantation: expression of the polymorphic mucin MUC-1 and adhesion molecules during the endometrial cycle. *Annals of the New York Academy of Sciences* **734** 103–121. (<https://doi.org/10.1111/j.1749-6632.1994.tb21739.x>)
- Ashworth CJ, Finch AM, Page KR, Nwagwu MO & McArdle HJ 2001 Causes and consequences of fetal growth retardation in pigs. *Reproduction* **58** 233–246.
- Baxter EM, Jarvis S, Palarea-Albaladejo J & Edwards SA 2012 The weaker sex? the propensity for male-biased piglet mortality. *PLoS One* **7** e30318. (<https://doi.org/10.1371/journal.pone.0030318>)
- Bayless KJ, Erikson DW, Frank JW, Joyce MM, Burghardt RC, Bazer FW & Johnson GA 2010 Recruitment and modulation of porcine endothelial progenitor cells by secreted phosphoprotein 1 (*SPP1*, osteopontin). *Biology of Reproduction* **83** 94. (<https://doi.org/10.1093/biolreprod/83.s1.94>)
- Bloor DJ, Metcalfe AD, Rutherford A, Brison DR & Kimber SJ 2002 Expression of cell adhesion molecules during human preimplantation embryo development. *Molecular Human Reproduction* **8** 237–245. (<https://doi.org/10.1093/molehr/8.3.237>)
- Bowen JA, Bazer FW & Burghardt RC 1996 Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and

- trophoblast in vivo. *Biology of Reproduction* **55** 1098–1106. (<https://doi.org/10.1095/biolreprod55.5.1098>)
- Burghardt RC, Johnson GA, Jaeger LA, Ka H, Garlow JE, Spencer TE & Bazer FW 2002 Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. *Cells, Tissues, Organs* **172** 202–217. (<https://doi.org/10.1159/000066969>)
- Burghardt RC, Burghardt JR, Taylor JD, Reeder AT, Nguen BT, Spencer TE, Bayless KJ & Johnson GA 2009 Enhanced focal adhesion assembly reflects increased mechanosensation and mechanotransduction at maternal-conceptus interface and uterine wall during ovine pregnancy. *Reproduction* **137** 567–582. (<https://doi.org/10.1530/REP-08-0304>)
- Campbell S, Swann HR, Seif MW, Kimber SJ & Aplin JD 1995 Cell adhesion molecules on the oocyte and preimplantation human embryo. *Human Reproduction* **10** 1571–1578. (<https://doi.org/10.1093/HUMREP/10.6.1571>)
- Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA & Yoshinaga K 2000 Embryo implantation. *Developmental Biology* **223** 217–237. (<https://doi.org/10.1006/dbio.2000.9767>)
- Dai J, Peng L, Fan K, Wang H, Wei R, Ji G, Cai J, Lu B, Li B, Zhang D *et al.* 2009 Osteopontin induces angiogenesis through activation of PI3K/AKT and ERK1/2 in endothelial cells. *Oncogene* **28** 3412–3422. (<https://doi.org/10.1038/onc.2009.189>)
- Delon I & Brown NH 2007 Integrins and the actin cytoskeleton. *Current Opinion in Cell Biology* **19** 43–50. (<https://doi.org/10.1016/j.ceb.2006.12.013>)
- Denker HW 1993 Implantation: a cell biological paradox. *Journal of Experimental Zoology* **266** 541–558. (<https://doi.org/10.1002/jez.1402660606>)
- Drickamer LC, Arthur RD & Rosenthal TL 1997 Conception failure in swine: importance of the sex ratio of a female's birth litter and tests of other factors. *Journal of Animal Science* **75** 2192–2196. (<https://doi.org/10.2527/1997.7582192x>)
- Drickamer LC, Rosenthal TL & Arthur RD 1999 Factors affecting the number of teats in pigs. *Journal of Reproduction and Fertility* **115** 97–100. (<https://doi.org/10.1530/jrf.0.1150097>)
- Dunlap KA, Erikson DW, Burghardt RC, White FJ, Reed KM, Farmer JL, Spencer TE, Magness RR, Bazer FW, Bayless KJ *et al.* 2008 Progesterone and placental increase secreted phosphoprotein one (SPP1 or osteopontin) in uterine glands and stroma for histotrophic and hematrophic support of ovine pregnancy. *Biology of Reproduction* **79** 983–990. (<https://doi.org/10.1095/biolreprod.108.071068>)
- Erikson DW, Burghardt RC, Bayless KJ & Johnson GA 2009a Secreted phosphoprotein 1 (SPP1, osteopontin) binds to integrin α 5 β 1 on porcine trophoblast cells and integrin α 5 β 1 on uterine luminal epithelial cells, and promotes trophoblast cell adhesion and migration. *Biology of Reproduction* **81** 814–825. (<https://doi.org/10.1095/biolreprod.109.078600>)
- Erikson DW, Burghardt RC, Bazer FW, Johnson GA & Bayless KJ 2009b Secreted phosphoprotein 1 (SPP1, osteopontin) stimulates adhesion and migration of circulating endothelial cells in pigs. *Biology of Reproduction* **81** 91. (<https://doi.org/10.1093/biolreprod/81.s1.91>)
- Erkens T, Van Poucke M, Vandesompele J, Goossens K, Van Zeveren A & Peelman LJ 2006 Development of a new set of reference genes for normalization of real-time RT-PCR data of porcine backfat and longissimus dorsi muscle, and evaluation with PPARGC1A. *Biomed Central Biotechnology* **6** 1–8. (<https://doi.org/10.1186/1472-6750-6-41>)
- Fassler R & Meyer M 1995 Consequences of lack of β 1 integrin gene expression in mice. *Genes and Development* **9** 1896–1908. (<https://doi.org/10.1101/gad.9.15.1896>)
- Fazleabas AT, Bell SC, Fleming S, Sun J & Lessey BA 1997 Distribution of integrins and the extracellular matrix proteins in the baboon endometrium during the menstrual cycle and early pregnancy. *Biology of Reproduction* **56** 348–356. (<https://doi.org/10.1095/biolreprod56.2.348>)
- Feinberg RF, Kliman HJ & Lockwood CJ 1991 Is oncofetal fibronectin a trophoblast glue for human implantation? *American Journal of Pathology* **138** 537–543.
- Finch AM, Anitpatis C, Pickard AR & Ashworth CJ 2002 Patterns of fetal growth within large white X landrace and Chinese Meishan gilt litters at three stages of gestation. *Reproduction, Fertility and Development* **14** 419–425. (<https://doi.org/10.1071/RD01131>)
- Ford SP, Vonnahme KA & Wilson ME 2002 Uterine capacity in the pig reflects a combination of uterine environment and conceptus genotype effects. *Journal of Animal Science* **80** E66–E73. (<https://doi.org/10.2527/animalsci2002.0021881200800ES10010x>)
- Foxcroft GR & Town SC 2004 Prenatal programming of postnatal performance – the unseen cause of variance. *Advances in Pork Production* **15** 4–10.
- Foxcroft GR, Dixon WT, Novak S, Putman CT, Town SC & Vinsky MDA 2006 The biological basis for prenatal programming of postnatal performance in pigs. *Journal of Animal Science* **84** E105–E112. (https://doi.org/10.2527/2006.8413_supplE105x)
- Frank JW, Seo H, Burghardt RC, Bayless KJ & Johnson GA 2017 ITGAV (α 5 integrin) binds SPP1 (osteopontin) to support trophoblast cell adhesion. *Reproduction* **153** 695–706. (<https://doi.org/10.1530/REP-17-0043>)
- Gallant ND, Michael KE & García AJ 2005 Cell adhesion Strengthening: contributions of adhesive area, integrin binding, and focal adhesion assembly. *Molecular Biology of the Cell* **16** 4329–4340. (<https://doi.org/10.1091/mbc.e05-02-0170>)
- Garlow JE, Ka H, Johnson GA, Burghardt RC, Jaeger LA & Bazer FW 2002 Analysis of osteopontin at the maternal-placental interface in pigs. *Biology of Reproduction* **66** 718–725. (<https://doi.org/10.1095/biolreprod66.3.718>)
- Germeyer A, Savaris RF, Jauckus J & Lessey B 2014 Endometrial β 3 integrin profile reflects endometrial receptivity defects in women with unexplained recurrent pregnancy loss. *Reproductive Biology and Endocrinology* **12** 53. (<https://doi.org/10.1186/1477-7827-12-53>)
- Giancotti FG & Ruoslahti E 1999 Integrin signaling. *Science* **285** 1028–1032. (<https://doi.org/10.1126/science.285.5430.1028>)
- Gonzalez TL, Sun T, Koepfel AF, Lee B, Wang ET, Farber CR, Rich SS, Sundheimer LW, Buttle RA, Chen YI *et al.* 2018 Sex differences in the late first trimester human placenta transcriptome. *Biology of Sex Differences* **9** 4. (<https://doi.org/10.1186/s13293-018-0165-y>)
- Górecki MT 2003 Sex ratio in litters of domestic pigs (Sus scrofa f. domestica Linnaeus, 1758). *Biology Letters* **40** 111–118.
- Guillomot M 1999 Changes in extracellular matrix components and cytochromes in the endometrium during goat implantation. *Placenta* **20** 339–345. (<https://doi.org/10.1053/plac.1998.0385>)
- Hernández SC, Hogg CO, Billon Y, Sanchez MP, Bidanel JP, Haley CS, Archibald AL & Ashworth CJ 2013 Secreted phosphoprotein 1 expression in endometrium and placental tissues of hyperprolific large white and Meishan gilts. *Biology of Reproduction* **88** 120. (<https://doi.org/10.1095/biolreprod.112.104679>)
- Hodivala-Dilke KM, Mchugh KP, Tsakiris DA, Rayburn H, Crowley D, Ullman-Cullere M, Ross FP, Collier BS, Teitelbaum S & Hynes RO 1999 β 3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *Journal of Clinical Investigation* **103** 229–238. (<https://doi.org/10.1172/JCI5487>)
- Huhn R, Tuchscherer A, Breite L, Grodzyski M & Huhn U 2002 Influence of base litter sex ratio of gilts on their subsequent reproductive performances. *Zuchtungskunde* **74** 56–69.
- Humphries JD, Byron A & Humphries MJ 2006 Integrin ligands at a glance. *Journal of Cell Science* **119** 3901–3903. (<https://doi.org/10.1242/jcs.03098>)
- Illera MJ, Cullinan E, Gui Y, Yuan L, Beyler SA & Lessey BA 2000 Blockade of the α 5 β 1 integrin adversely affects implantation in the mouse. *Biology of Reproduction* **62** 1285–1290. (<https://doi.org/10.1095/biolreprod62.5.1285>)
- Illera MJ, Lorenzo PL, Gui YT, Beyler SA, Apparao KBC & Lessey BA 2003 A role for α 5 β 1 integrin during implantation in the rabbit model. *Biology of Reproduction* **68** 766–771. (<https://doi.org/10.1093/biolreprod/68.3.766>)
- Imakawa K, Imai M, Sakai A, Suzuki M, Nagaoka K, Sakai S, Lee SR, Chang KT, Echtenkamp SE & Christenson RK 2006 Regulation of conceptus adhesion by endometrial CXCL12 chemokines during the implantation period in sheep. *Molecular Reproduction and Development* **73** 850–858. (<https://doi.org/10.1002/mrd.20496>)
- Irving JA & Lala PK 1995 Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF- β , IGF-II, and IGF-BP-1. *Experimental Cell Research* **217** 419–427. (<https://doi.org/10.1006/excr.1995.1105>)
- Johnson GA, Burghardt RC, Spencer TE, Newton GR, Ott TL & Bazer FW 1999 Ovine osteopontin: II. Osteopontin and α 5 β 1 integrin expression in the uterus and conceptus during the periimplantation period. *Biology of Reproduction* **61** 892–899. (<https://doi.org/10.1095/biolreprod61.4.892>)

- Johnson GA, Bazer FW, Jaeger LA, Ka H, Garlow JE, Pfarrer C, Spencer TE & Burghardt RC 2001 Muc-1, integrin, and osteopontin expression during the implantation cascade in sheep. *Biology of Reproduction* **65** 820–828. (<https://doi.org/10.1095/biolreprod65.3.820>)
- Kalisch-Smith JL, Simmons DG, Dickinson H & Moritz KM 2017 Review: sexual dimorphism in the formation, function and adaptation of the placenta. *Placenta* **54** 10–16. (<https://doi.org/10.1016/j.placenta.2016.12.008>)
- Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A, Osteen K, Taylor RN, Lessey BA & Giudice LC 2002 Global gene profiling in human endometrium during the window of implantation. *Endocrinology* **143** 2119–2138. (<https://doi.org/10.1210/endo.143.6.8885>)
- Keys JL, King GJ & Kennedy TG 1986 Increased uterine permeability at the time of embryonic attachment in the pig. *Biology of Reproduction* **34** 405–411. (<https://doi.org/10.1095/biolreprod34.2.405>)
- Kimmins S & MacLaren LA 1999 Cyclic modulation of integrin expression in bovine endometrium. *Biology of Reproduction* **61** 1267–1274. (<https://doi.org/10.1095/biolreprod61.5.1267>)
- King DP, Burman A, Gold S, Shaw AE, Jackson T & Ferris NP 2011 Integrin sub-unit expression in cell cultures used for the diagnosis of foot-and-mouth disease. *Veterinary Immunology and Immunopathology* **140** 259–265. (<https://doi.org/10.1016/j.vetimm.2011.01.008>)
- Koketsu Y, Tani S & Iida R 2017 Factors for improving reproductive performance of sows and herd productivity in commercial breeding herds. *Porcine Health Management* **3** 1. (<https://doi.org/10.1186/s40813-016-0049-7>)
- Lamberson WR, Blair RM, Rohde Parfet KA, Day BN & Johnson RK 1988 Effect of sex ratio of the birth litter on subsequent reproductive performance of gilts. *Journal of Animal Science* **66** 595–598. (<https://doi.org/10.2527/jas1988.663595x>)
- Lessey BA, Castelbaum AJ, Buck CA, Lei Y, Yowell CW & Sun J 1994 Further characterization of endometrial integrins during the menstrual cycle and in pregnancy. *Fertility and Sterility* **62** 497–506. ([https://doi.org/10.1016/S0015-0282\(16\)56937-4](https://doi.org/10.1016/S0015-0282(16)56937-4))
- Lin H, Wang X, Liu G, Fu J & Wang A 2007 Expression of α V and β 3 integrin subunits during implantation in pig. *Molecular Reproduction and Development* **74** 1379–1385. (<https://doi.org/10.1002/mrd.20732>)
- Marrable AW 1971 *The Embryonic Pig: A Chronological Account*. London: Pitman Medical.
- Montiel JF, Kaune H & Maliqueo M 2013 Maternal-fetal unit interactions and eutherian neocortical development and evolution. *Frontiers in Neuroanatomy* **7** 22. (<https://doi.org/10.3389/fnana.2013.00022>)
- Nygard AB, Jørgensen CB, Cirera S & Fredholm M 2007 Selection of reference genes for gene expression studies in pig tissues using SYBR green qPCR. *Biomed Central Molecular Biology* **8** 1–6. (<https://doi.org/10.1186/1471-2199-8-67>)
- Pettigrew JE, Cornelius SG, Moser RL, Heeg TR, Hanke HE, Miller KP & Hagen CD 1986 Effects of oral doses of corn oil and other factors on preweaning survival and growth of piglets. *Journal of Animal Science* **62** 601–612. (<https://doi.org/10.2527/jas1986.623601x>)
- Qin L, Wang YL, Bai SX, Ji SH, Qiu W, Tang S & Piao YS 2003 Temporal and spatial expression of integrins and their extracellular matrix ligands at the maternal-fetal interface in the rhesus monkey during pregnancy. *Biology of Reproduction* **69** 563–571. (<https://doi.org/10.1095/biolreprod.103.015198>)
- Rashev P, Georgieva R & Rees D 2005 Expression of α 5 β 1 integrin and fibronectin during early pregnancy in pigs. *Folia Biologica* **51** 121–125.
- Rosenfeld CS 2015 Sex-specific placental responses in fetal development. *Endocrinology* **156** 3422–3434. (<https://doi.org/10.1210/en.2015-1227>)
- Ruck P, Marzusch K, Kaiserling E, Horny HP, Dieltl J, Geiselhart A, Handgretinger R & Redman CW 1994 Distribution of cell adhesion molecules in decidua of early human pregnancy. An immunohistochemical study. *Laboratory Investigation* **71** 94–101.
- Schultz JF & Armant DR 1995 β 1- and β 3-class integrins mediate fibronectin binding activity at the surface of developing mouse peri-implantation blastocysts. *Journal of Biological Chemistry* **270** 11522–11531. (<https://doi.org/10.1074/jbc.270.19.11522>)
- Schultz JF, Mayernik L, Rout UK & Armant DR 1997 Integrin trafficking regulates adhesion to fibronectin during differentiation of mouse peri-implantation blastocysts. *Developmental Genetics* **21** 31–43. ([https://doi.org/10.1002/\(SICI\)1520-6408\(1997\)21:1<31::AID-DVG4>3.0.CO;2-9](https://doi.org/10.1002/(SICI)1520-6408(1997)21:1<31::AID-DVG4>3.0.CO;2-9))
- Schwartz MA & Assoian RK 2001 Integrins and cell proliferation: regulation of cyclin-dependent kinases via cytoplasmic signaling pathways. *Journal of Cell Science* **114** 2553–2560.
- Shiokawa S, Yoshimura Y, Nagamatsu S, Sawa H, Hanashi H, Koyama N, Katsumata Y, Nagai A & Nakamura Y 1996 Function of β 1 integrins on human decidua cells during implantation. *Biology of Reproduction* **54** 745–752. (<https://doi.org/10.1095/biolreprod54.4.745>)
- Shiokawa S, Yoshimura Y, Sawa H, Nagamatsu S, Hanashi H, Sakai K, Ando M & Nakamura Y 1999 Functional role of Arg-Gly-Asp (RGD)-binding sites on β 1 integrin in embryo implantation using mouse blastocysts and human decidua. *Biology of Reproduction* **60** 1468–1474. (<https://doi.org/10.1095/biolreprod60.6.1468>)
- Steinhauser CB, Bazer FW, Burghardt RC & Johnson GA 2017 Expression of progesterone receptor in the porcine uterus and placenta throughout gestation: correlation with expression of uteroferrin and osteopontin. *Domestic Animal Endocrinology* **58** 19–29. (<https://doi.org/10.1016/j.domaniend.2016.07.002>)
- Stenhouse C 2018 Investigating the Porcine Feto-Maternal Interface Throughout Gestation: Associations with Foetuses of Different Size and Sex. PhD Thesis. University of Edinburgh.
- Stenhouse C, Tennant P, Duncan WC & Ashworth CJ 2018b Doppler ultrasound can be used to monitor umbilical arterial blood flow in lightly sedated pigs at multiple gestational ages. *Reproduction, Fertility, and Development* **30** 1402–1411. (<https://doi.org/10.1071/RD17298>)
- Stenhouse C, Hogg CO & Ashworth CJ 2018a Associations between fetal size, sex and placental angiogenesis in the pig. *Biology of Reproduction* **100** 239–252. (<https://doi.org/10.1093/biolre/iay184>)
- Stephens LE, Sutherland AE, Klimanskaya IV, Andrieux A, Meneses J, Pedersen RA & Damsky CH 1995 Deletion of β 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. *Genes and Development* **9** 1883–1895. (<https://doi.org/10.1101/gad.9.15.1883>)
- Sutherland AE, Calarco PG & Damsky CH 1993 Developmental regulation of integrin expression at the time of implantation in the mouse embryo. *Development* **119** 1175–1186.
- Vallet JL & Freking BA 2007 Differences in placental structure during gestation associated with large and small pig fetuses. *Journal of Animal Science* **85** 3267–3275. (<https://doi.org/10.2527/jas.2007-0368>)
- Vallet JL, McNeel AK, Miles JR & Freking BA 2014 Placental accommodations for transport and metabolism during intra-uterine crowding in pigs. *Journal of Animal Science and Biotechnology* **5** 1.
- Vélez C, Williamson D, Riesco O, Martín P, García M, Yaful G & Koncurat M 2015 Estrogens, progesterone and integrin-fibronectin interaction during porcine placentation. *Placenta* **36** 500. (<https://doi.org/10.1016/j.placenta.2015.01.484>)
- Vélez C, Barbeito C & Koncurat M 2017 α v β 3 integrin and fibronectin expressions and their relation to estrogen and progesterone during placentation in swine. *Biotechnic and Histochemistry* **7** 1–7.
- Vonnahme KA, Wilson ME & Ford SP 2001 Relationship between placental vascular endothelial growth factor expression and placental/endometrial vascularity in the pig. *Biology of Reproduction* **64** 1821–1825. (<https://doi.org/10.1095/biolreprod64.6.1821>)
- Wang Y, Yan W, Lu X, Qian C, Zhang J, Li P, Shi L, Zhao P, Fu Z, Pu P *et al.* 2011 Overexpression of osteopontin induces angiogenesis of endothelial progenitor cells via the α v β 3/PI3K/AKT/eNOS/NO signaling pathway in glioma cells. *European Journal of Cell Biology* **90** 642–648. (<https://doi.org/10.1016/j.ejcb.2011.03.005>)
- White FJ, Ross JW, Joyce MM, Geisert RD, Burghardt RC & Johnson GA 2005 Steroid regulation of cell specific secreted phosphoprotein 1 (osteopontin) expression in the pregnant porcine uterus. *Biology of Reproduction* **73** 1294–1301. (<https://doi.org/10.1095/biolreprod.105.045153>)
- White FJ, Burghardt RC, Hu J, Joyce MM, Spencer TE & Johnson GA 2006 Secreted phosphoprotein 1 (osteopontin) is expressed by stromal macrophages in cyclic and pregnant endometrium of mice, but is induced by estrogen in luminal epithelium during conceptus attachment for implantation. *Reproduction* **132** 919–929. (<https://doi.org/10.1530/REP-06-0068>)
- Widdowson EM 1971 Intra-uterine growth retardation in the pig I Organ Size and cellular development at birth and after growth to maturity. *Biology of the Neonate* **19** 329–340. (<https://doi.org/10.1159/000240427>)
- Wise T, Roberts AJ & Christenson RK 1997 Relationships of light and heavy fetuses to uterine position, placental weight, gestational age, and fetal

- cholesterol concentrations. *Journal of Animal Science* **75** 2197–2207. (<https://doi.org/10.2527/1997.7582197x>)
- Wu G, Bazer FW, Burghardt RC, Johnson GA, Kim SW, Li XL, Satterfield MC & Spencer TE 2010 Impacts of amino acid nutrition on pregnancy outcome in pigs: mechanisms and implications for swine production. *Journal of Animal Science* **88** E195–E204. (<https://doi.org/10.2527/jas.2009-2446>)
- Yelich JV, Pomp D & Geisert RD 1997 Detection of transcripts for retinoic acid receptors, retinol-binding protein, and transforming growth factors during rapid trophoblastic elongation in the porcine conceptus. *Biology of Reproduction* **57** 286–294. (<https://doi.org/10.1095/biolreprod57.2.286>)
- Yoshimura Y, Shiokawa S, Nagamatsu S, Hanashi H, Sawa H, Koyama N, Katsumata Y & Nakamura Y 1995 Effects of beta-1 integrins in the process of implantation. *Hormone Research* **44** 36–41. (<https://doi.org/10.1159/000184659>)
- Zheng J, Wen YX, Austin JL & Chen DB 2006 Exogenous nitric oxide stimulates cell proliferation via activation of a mitogen-activated protein kinase pathway in ovine fetoplacental artery endothelial cells. *Biology of Reproduction* **74** 375–382. (<https://doi.org/10.1095/biolreprod.105.043190>)
- Zygmunt M, Boving B, Wienhard J, Münstedt K, Braems G, Bohle RM & Lang U 1997 Expression of cell adhesion molecules in the extravillous trophoblast is altered in IUGR. *American Journal of Reproductive Immunology* **38** 295–301. (<https://doi.org/10.1111/j.1600-0897.1997.tb00518.x>)

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