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Running Title: Porcine fetal size and ovarian development

Associations between foetal size and ovarian development in the pig

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ABSTRACT

It is estimated that intra-uterine growth restricted piglets represent 25% of the total number of piglets born. Growth restricted female piglets have impaired reproductive performance postnatally, however, when during gestation this phenotype arises is not known. With this study, the aim was to improve the understanding of foetal ovarian development in normal and small foetuses throughout gestation. Female Large White X Landrace foetuses were obtained at gestational day (GD) 45, 60 and 90 (n = 5-6 litters/GD). Histological analysis of GATA4 stained foetal ovaries at GD60 and 90 indicated there were fewer primary follicles ($P \leq 0.05$) in the foetuses weighing the least compared to those with a weight similar to the mean for the litter (CTMLW) at GD90. Plasma oestradiol concentrations were less in the foetuses with lesser weights compared with greater weight foetuses at GD90 ($P \leq 0.05$). The RNA was extracted from ovaries of the lesser weight and CTMLW foetuses at GD45, 60 and 90 and qPCR was performed to quantify relative abundance of 12 candidate mRNAs for which encoded proteins modulate ovarian function and development. Gestational changes in relative abundances of *CD31*, *PTGFR*, *SPP1* and *VEGFA* mRNA transcripts were observed. Relative abundance of *KI67* (P = 0.066) and *P53* ($P \leq 0.05$) was less in ovaries of the

lesser weight compared to CTMLW foetuses at GD60. There was a lesser relative abundance of *PTGFR* mRNA transcript in ovaries from the foetuses with lesser weight compared to CTMLW foetuses at GD45 and 60 ($P \leq 0.05$). These findings indicate that postnatal differences in reproductive potential of growth restricted females are programmed early in gestation. It is hoped that further investigation will improve the understanding of the relationship between prenatal reproductive development and postnatal reproductive performance.

Keywords: Foetal Growth; Intrauterine Growth Restriction (IUGR); Porcine; Ovary.

1. Introduction

The number of primordial follicles present in the ovary at birth is considered to define a female's reproductive potential, a hypothesis first suggested in the 19th century (Waldeyer, 1870). Numerous processes must occur in the ovary during foetal development for there to be adequate formation of the pool of primordial follicles including proliferation and apoptosis of the germ cells, formation of oogonial nests followed by follicular formation and growth, accompanied by initiation of meiosis.

Impaired foetal growth is associated with impaired reproductive potential post-puberty, in both sexes and in multiple species. In an experimentally induced model of intrauterine growth restriction (IUGR), there were less ovarian follicles during the late gestation period in sheep foetuses (Da Silva et al., 2002). The lesser follicle numbers in ewes with a lesser foetal body weight included mainly the primordial follicle pool, suggesting that the impaired ovarian function observed postnatally may be associated with defects in foetal ovarian development.

Significant variation in birth weight is observed in piglets, with small outliers being a problem to the industry. It is estimated that IUGR piglets contribute to 25% of the total number of piglets born (Wu et al., 2010). Many definitions for IUGR exist including piglets weighing less

than 1.1 kg at birth, weighing less than two standard deviations of the mean body weight for age, the smallest of each litter or a small weight statistical outlier from the population, or weighing less than two-thirds of the mean litter weight (Ashworth et al., 2001; Royston et al., 1982; van der Lende et al., 1990; Wu et al., 2010). It has been suggested that IUGR in the piglet may be programmed from an early stage of gestation, with marked within-litter variation in foetal size observed from as early as gestational day 30 to 35 (Finch et al., 2002; Foxcroft et al., 2006; Foxcroft and Town, 2004; Pettigrew et al., 1986; Wise et al., 1997).

At birth, IUGR gilts have delayed initiation of follicular development, with more primordial follicles, accompanied by fewer primary and secondary follicles compared to normally developing littermates (Da Silva-Buttkus et al., 2003). Gilts with lesser growth rates are older when first mating occurs (Tummaruk et al., 2000), which results in fewer litters produced by sows in pork production enterprises. It has been suggested that in addition to having an effect on reproductive performance during their first pregnancy, gilts which have lesser birth weights will also have impaired first parity reproductive performance (lesser pregnancy rates and litter sizes) and also at their second parity (Hoving et al., 2010).

Dysregulation of the foetal hypothalamic-pituitary-gonadal axis can alter the production of androgens and oestrogens, in turn causing defective reproductive organogenesis and altered development of the foetus (Brooks and Thomas, 1995). In mammals, an aberrant oestrogen milieu during foetal development can have marked effects on postnatal ovarian function (Abbott et al., 2006). Considering the functions of oestrogens in the regulation of oestrous cycles, oestradiol concentrations in gilts during the prepubertal period can be used as a biomarker for reproductive efficiency (Steel et al., 2018).

Impaired reproductive performance is one of the primary reasons for the removal of sows from commercial herds (Sasaki and Koketsu, 2011). Considering the prevalence of small birthweight piglets, and the severity of this reproductive phenotype, it is important that there be improvement in understanding of the mechanisms regulating foetal ovarian development to improve reproductive efficiency. Whilst in previous studies the temporal changes in follicle types during gestation have been elucidated (Oxender et al., 1979; Pontelo et al., 2018), there is a limited understanding of the expression profiles of genes, as indicated by relative abundance of mRNA transcripts, associated with ovarian development and function.

There are marked changes in the structure of the foetal ovary during gestation that affect female fertility postnatally. In the present study, the temporal relative abundance profiles of mRNA transcripts with central functions in apoptosis, proliferation and the ovarian extracellular matrix was determined. Even though it is widely recognized that angiogenesis is essential for organ development and growth, and that angiogenesis has important effects in the postnatal ovary, little is known regarding vascularisation of the foetal ovary. Considering this, the encoded proteins of several of the candidate genes investigated in the present study have central functions in the regulation of angiogenesis.

In the present study, the aim was to improve understanding of temporal changes in ovarian gene expression, as indicated by relative abundances of mRNA transcripts, and histology in foetuses with relatively average and lesser than average birth weights as compared with the entire litter. It was hypothesised that the developmental trajectory of ovarian development of the foetuses with lesser weights deviates from that of ovaries from average-sized female foetuses.

2. 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.

2.1. Experimental animals and sample collection

Large White X Landrace gilts (age 11 - 14 months) were observed daily for signs of oestrus and were housed in groups of 6 to 8 animals per pen. Oestrous cyclicity and ovarian function were controlled in accordance with routine normal practice at The Roslin Institute Large Animal Unit. All gilts were inseminated twice daily for the duration of oestrus with semen from one of four Large White sires. The sires used were equally distributed between gilts at the different gestational days (GD) of interest to minimize any effect of sire. The first day of insemination was assigned as GD0. Gilts were humanely killed 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 at GD45, GD60 and GD90 (n = five – eight gilts/GD). Immediately before euthanasia, cardiac puncture was performed using an EDTA coated syringe to collect maternal blood from five gilts of the GD90 group.

Following confirmation of death, mid-ventral incisions were made so that there was access to the

reproductive tract. The tract was removed from the body cavity and placed in a dissecting tray. Ovaries were removed from the uterus and the number of corpora lutea was quantified. The percentage prenatal survival was calculated by dividing the number of live foetuses by the number of corpora lutea and multiplying this value by 100. Both uterine horns were dissected, from the ovary towards the cervix. Foetal sex was determined morphologically. Cardiac puncture was performed using an EDTA coated syringe to collect blood samples from the female foetuses with the relatively lesser, average (CTMLW) and relatively greater weights when there was consideration of the individual foetal weights for the entire litter at GD90 (*n* = five litters; Supplementary Table 1). Plasma was obtained from the maternal and foetal blood samples by centrifugation and samples were stored at -20 °C until required. At GD45, GD60 and GD90 (*n* = five or six litters/GD), ovaries from the foetuses that had relatively lesser, CTMLW and relatively greater weights were dissected, weighed (GD60 and GD90), and one ovary from each foetus was snap frozen in liquid nitrogen and stored at -80 °C until relevant evaluations occurred. The other ovary was fixed in Bouin's for histological analysis.

Comparisons were made between the female foetuses that had relatively lesser, CTMLW and relatively

greater weights for plasma oestradiol concentrations and ovarian weight. Considering that the majority of litters have a negatively skewed distribution of birth weights (Milligan et al., 2002; Quesnel et al., 2008), and considering the greater prevalence of the lesser birth weight outliers, the focus for the remainder of the analyses was the lighter and CTMLW foetal comparisons.

2.2. Histological analysis

Ovarian samples from GD60 (*n* = seven CTMLW foetuses; eight lesser weight foetuses) and GD90 (*n* = six from both the CTMLW and lesser weight foetuses) were used. Whole ovaries were fixed with Bouin's overnight at room temperature and changed daily for approximately 1 week in 70% ethanol (Genta Medical). The ovaries were then transferred into labelled tissue processing cassettes (Simport) and processed using a tissue processor (ASP3005, Leica) by passing through graded ethanol (70%, 95%, and 99%; Genta Medical) and xylene (Genta Medical). The samples were embedded in paraffin wax (Fisher Scientific), and 5 μm sections were cut and placed on polysine microscope slides (Fisher Scientific).

Following dewaxing and heat-induced epitope retrieval in 0.01 M sodium citrate (Vector Laboratories), endogenous peroxidase activity was blocked by incubating slides with 0.3% hydrogen peroxide (Sigma Aldrich) in

methanol. Non-specific binding sites were blocked by incubation with normal goat serum (Vectastain Elite ABC kit; Vector Laboratories). Sections were incubated with a primary antibody for GATA binding protein 4 (GATA4; sc-9053; Santa Cruz) at a 1:200 dilution, anti-CD31 antibody (ab28364; Abcam) at a 1:100 dilution (GD90 ovaries only) or with rabbit immunoglobulin G (RIgG; Vector Laboratories; equivalent total protein concentration) as a negative control. Sections were stained with GATA4 as this protein is exclusively expressed by somatic cells, and not by germ cells (Mccoard et al., 2001). The slides were incubated in a humidified chamber at 4°C overnight, washed in phosphate buffered saline (PBS), and incubated for 30 min at room temperature with a biotinylated anti-rabbit IgG secondary antibody (Vectastain Elite ABC kit; Vector Laboratories) at a dilution of 1:200 in PBS containing 1.5% normal goat serum. Sections were incubated with Vectastain Elite ABC reagent (Vectastain Elite ABC kit; Vector Laboratories) for 30 min, before incubation with the Novared peroxidase substrate (Vector Laboratories) for 5 min. Sections were counterstained with haematoxylin and dehydrated in a graded series of ethanol and xylene (70%, 95%, and 99% ethanol; 99% ethanol 1:1 with xylene, and absolute xylene; Genta Medical). The

sections were imaged using the NanoZoomer slide scanner (Hamamatsu).

2.2.1. Image analysis

All image analyses were performed using ImageJ. Six non-overlapping images from both the GATA4 and CD31 stained sections were taken at x 20 magnification. For each ovary, two GATA4 stained sections were analysed which were a minimum of 15 serial sections apart from one another from the middle of the ovary. Using the GATA4 stained sections, the number of oogonia (GD60 and GD90), primordial (GD60 and GD90), and primary (GD90) follicles were quantified within each image. The data were then expressed as number of oogonial nest/follicles per 100000μM^2. The number of oogonia per germ cell nest was counted. Only a few primary and secondary follicles were observed at GD60 and GD90, respectively, so further analyses on these follicle types at these gestational days could not be performed.

2.3. Plasma oestradiol quantification

Oestradiol concentrations were determined in maternal plasma samples at GD90 (*n* = 5) and in foetal plasma samples from the relatively lesser, CTMLW and relative greater weight female foetuses at GD90 (*n* = 5 litters) in a single ELISA validated for use with samples of pigs (Abscitech; EK0373), as per the manufacturer's

instructions. The detection range of the assay was 20 pg/ml to 1,600 pg/ml, with a sensitivity of 25 pg/ml.

2.4. Analysis of relative abundances of mRNA transcripts for candidate genes using qPCR

The relative abundance of mRNA transcripts for candidate genes was investigated using qPCR in ovarian samples from the foetuses that were of relatively lesser weights and CTMLW at GD45, GD60 and GD90 (*n* = five – six litters/GD).

2.4.1. Total RNA extraction and cDNA synthesis

The RNA was extracted from 20 to 50 µg of snap-frozen ovarian samples as described previously (Stenhouse et al., 2019, 2018a, 2018b). The RNA was quantified, and the quality assessed spectrophotometrically using a Nanodrop ND-1000 (Labtech International Ltd.) and electrophoretically using a Tapestation 2200 (Agilent Technologies; RNA Integrity Number Equivalent - RINe; 9.209 ± 0.009). Extracted RNA was stored at -80°C until required.

Complementary DNA (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 assess for genomic contamination and all cDNA was stored at -20ºC until required.

2.4.2. Quantification of relative abundance of mRNA transcripts for candidate genes in ovarian samples

Quantitative PCR was performed using a Stratagene MX3000 instrument using SensiFAST® SYBR Lo-ROX (Bioline) utilising cDNA from foetal ovaries at GD45, GD60 and GD90. All qPCRs were conducted at an annealing temperature of 60ºC and dissociation curves consisting of single peaks were generated. The relative abundance of mRNA transcripts for candidate genes was quantified: BCL-2-associated X protein (*BAX*) (Zhao et al., 2014), B-cell lymphoma 2 (*BCL2*), platelet endothelial cell adhesion molecule (*CD31*), Doublesex and Mab-3 Related Transcription Factor 1 (*DMRT1*), GATA Binding Protein 4 (*GATA4*), hypoxia inducible factor 1 alpha subunit (*HIF1A*) (Oliver et al., 2011), insulin like protein 3 (*INSL3*), *KI67*, tumour suppressor protein 53 (*P53*), prostaglandin F2 α receptor (*PTGFR*) (Kaczynski and Waclawik, 2013) , secreted phosphoprotein 1 (*SPP1*) (Hernández et al., 2013), and vascular endothelial growth factor A (*VEGFA*) (Oliver et al., 2011). Two reference genes were used: TATA box binding protein 1 (*TBP1*) (Nygard et al., 2007) and Topoisomerase II Beta (*TOP2B*) (Erkens et al., 2006). These reference genes were identified as having stable

mRNA in ovarian samples by geNORM V3.5 (Ghent University Hospital, Centre for Medical Genetics). The primer sequences for all genes investigated are detailed in Supplementary Table 2.

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 2 µl of 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. SensiFAST® SYBR Lo-ROX supermix (5 µl), 10 µM forward and reverse primer stock (0.4 µl each) and water (2.2 µl).

Data were analysed using qbase+ software V3.0 (Biogazelle). A target and procedural specific strategy was utilised and the results, normalised to the geometric mean of two reference genes using the $2^{-\Delta\Delta CT}$, were scaled to the minimum sample. The mean slope, intercept, PCR efficiency and R^2 values are detailed in Supplementary Table 3.

2.5. Statistical analysis

All statistical analyses were performed using GenStat 13.1 (VSN International Ltd.). Mean values were calculated for each individual sample for each variable investigated and the normality of the distribution of the data

was assessed using an Anderson-Darling test. If there was a *P* value of <0.05, the data were not considered to have a normal distribution. Log10 and square root transformations were conducted to achieve normality of the distribution of the data where required. Outlier data points identified using a ROUT outlier test were excluded.

Where data had a normal distribution, ANOVA for GD or foetal size was conducted, with a block for gilt to account for the common maternal environment. When results with use of an ANOVA indicated there was significance, a *post-hoc* Tukey test was performed. Where data were not normally distributed, the Kruskal-Wallis and Mann Whitney tests were performed where appropriate. Analyses for foetal size were performed overall and within GD of interest. A two-way ANOVA with a block for gilt to account for the common maternal environment was performed to assess the presence of GD by foetal size interactions. To determine associations between litter size, percentage prenatal survival and percentage of males in the litter, and plasma oestradiol, Pearson's correlations were performed. Pearson's correlations were also performed for the comparison of foetal and ovarian weight. In all cases, significance was considered when there were mean differences with a *P*<0.05, with there being

considered to be a trending towards differences when the *P* was > 0.05 < 0.1 and not significant when *P*>0.1.

3. Results

3.1. Ovarian weight associations with foetal size at GD60 or GD90

Paired ovarian weight (Fig. 1A) and paired ovarian weight as a percentage of foetal weight (Fig. 1B) were not different between the female foetuses with the relatively lesser, CTMLW and relatively greater weights at GD60 or GD90. Paired ovarian weight (*P*<0.001; Fig. 1A) was greater at GD90 compared to GD60. In contrast, paired ovarian weight as a percentage of foetal weight (*P*<0.01) was less at GD90 compared to GD60. Results from regression analyses indicated there was a positive association between paired ovarian and foetal weight at GD60 (*P*<0.05; *R*2 = 0.370; Fig. 1C), but not GD90 (Fig. 1D). There were no associations between ovarian weight as a percentage of body and foetal weights at GD60 or GD90 (data not presented).

3.2. Composition of follicular pool in ovaries from the foetuses with lesser weight compared to the CTMLW at GD90

The GATA4 stained ovaries were used for quantification of oogonia and follicles in ovaries collected

396 at GD60 and GD90 ovaries (Supplementary Fig. 1).
397 Numbers of oogonial nests (Fig. 1E), primordial follicles
398 (Fig. 1F) and oogonia per germ cell nest (Fig. 1H) were
399 affected by gestational day (P<0.05) but not by foetal size.
400 In contrast, there were fewer primary follicles in the
401 foetuses with lesser weight compared to those that were
402 CTMLW at GD90 (Fig. 1G; P<0.05).

403 *3.3. Foetal plasma oestradiol concentration associations*
404 *with foetal size at GD90*

405 There were no associations between maternal
406 plasma oestradiol concentrations and number of live
407 foetuses (Fig. 2A). There was a trend towards an inverse
408 association between maternal plasma oestradiol
409 concentration and percentage prenatal survival at GD90
410 (R^2 = 0.6702; P = 0.09; Fig. 2B). Plasma oestradiol
411 concentrations were less in the lesser compared to the
412 greater weight female foetuses at GD90 ($P \leq 0.05$; Fig.
413 2C).

414 *3.4. Temporal changes in relative abundances of ovarian*
415 *CD31, PTGFR, SPP1 and VEGFA mRNA transcripts*

416 There were temporal changes in relative
417 abundance of mRNA transcripts of several candidate
418 genes investigated in ovaries collected at GD45, GD60
419 and GD90 (Table 1). There was a lesser relative
420 abundance of *CD31* mRNA transcript in ovaries between

GD60 and GD90 ($P \leq 0.05$; Table 1). Immunohistochemical evaluations for CD31 protein in ovaries at GD90 confirmed that CD31 is exclusively present in endothelial cells in the foetal ovary (Supplementary Fig. 2). Greater relative abundances of ovarian *PTGFR* mRNA at GD90 than GD60 ($P \leq 0.001$; Table 1). In contrast, there was a lesser relative abundance of ovarian *SPP1* mRNA transcript at GD60 than GD90 ($P \leq 0.01$; Table 1). There was a trend towards a gestational day effect on the relative abundance of *VEGFA* mRNA ($P = 0.086$; Table 1). There were no temporal changes in the relative abundance of *BAX*, *BCL2*, *DMRT1*, *GATA4*, *HIF1A*, *INSL3*, *KI67* or *P53* mRNA transcripts (Table 1).

3.5. Foetal size associations with relative abundance of KI67, P53 and PTGFR mRNA transcripts

The relative abundance of mRNA transcripts for the candidate genes were quantified and compared between ovaries from the foetuses that had relatively lesser weights, and CTMLW foetuses within GD (Fig. 3). At GD60, there was a trend towards a lesser relative abundance of *KI67* mRNA transcript in ovarian samples from the foetuses that had relatively lesser weights as compared with CTMLW ($P = 0.066$; Fig. 3I). Similarly, there was a lesser relative abundance of *P53* mRNA transcript

446 in ovarian samples from foetuses that had relatively lesser
447 weights than CTMLW at GD60 ($P \leq 0.05$; Fig. 3J). The
448 relative abundance of *PTGFR* mRNA transcript was less
449 in ovaries of foetuses that had relatively lesser weights
450 than the foetuses with CTMLW at both GD45 and GD60
451 ($P \leq 0.05$; Fig. 3K). There were GD by foetal size
452 interactions for *DMRT1* ($P < .05$; Fig. 3E) and *PTGFR*
453 ($P = 0.01$; Fig. 3K) mRNA transcripts. There were no other
454 GD x foetal size interactions. There were associations
455 between foetal size and relative abundances of ovarian
456 *BAX* (Fig. 3A), *BCL2* (Fig. 3B), *CD31* (Fig. 3D), *GATA4*
457 (Fig. 3F), *HIF1A* (Fig. 3G), *INSL3* (Fig. 3H), *SPP1* (Fig. 3L)
458 or *VEGFA* (Fig. 3M) mRNA transcripts.

459 ## 4. Discussion

460 Improved understanding of the mechanisms and
461 timing of developmental changes in the foetal ovary is
462 important for improving knowledge of how female pigs
463 reach their reproductive potential postnatally. This study
464 was conducted to determine whether there were
465 differences in foetal ovarian gene expression, as evaluated
466 by determination of relative abundances of mRNA
467 transcripts for genes of interest, between littermate
468 foetuses of different size from as early as GD45.

469 Dysregulation of the hypothalamic-pituitary-gonadal
470 axis can alter the production of androgens and oestrogens,

in turn causing defective reproductive organogenesis and altered development of the foetus (Brooks and Thomas, 1995). The lesser circulating oestradiol concentrations on GD90 in the lesser compared to greater weight foetuses indicates there needs to be further investigation into the prenatal development of the hypothalamus and pituitary in growth-restricted pig foetuses at multiple stages of gestation.

Primordial germ cells have been observed in the foetal pig ovary from as early as GD18. By GD30, germ cells are undergoing proliferation (Black and Erickson, 1968) which allows for an increase to maximal numbers by GD50 (Black and Erickson, 1968). Considering the marked differences that have been reported regarding variation in foetal size of pigs from this early stage of gestation (Finch et al., 2002; Foxcroft et al., 2006; Foxcroft and Town, 2004; Pettigrew et al., 1986; Wise et al., 1997), and the marked developmental changes of the ovary during early and mid-gestation, it was hypothesized that that there would be differences in relative abundances of mRNA transcripts in ovaries for candidate genes involved in extracellular-matrix remodelling, proliferation and apoptosis as gestational stages advanced and between littermates of different weight.

The SPP1 protein, also known as osteopontin, is an extra-cellular matrix (ECM) protein which functions by binding to integrin receptors present on the cell surface to promote cellular adhesion and communication (Humphries, 2006). As indicated by the follicular counts in the present study (Figure 1) and evaluations of GATA4 stained sections (Supplementary Figure 1), there were marked changes in the structure of the ovary during gestation. There are these developmental changes in the ovary so that essential functions can occur that are related to female fertility postnatally. Considering the important functions of this extracellular matrix protein in other tissues (Fisher et al., 2001; Johnson et al., 2014, 2003), the gestational changes in relative abundance of *SPP1* mRNA transcript were expected. Further studies should be performed to characterise the functions of SPP1, integrins, and other ligands of the integrin receptors such as fibronectin, in the foetal ovary, improving the understanding of the mechanisms involved in foetal ovarian development.

In the current study, there was a marked decrease in relative abundance of *KI67* mRNA transcript in ovaries of the foetuses with lesser weights compared to the CTMLW at GD60. There was a similar pattern at GD45 although there was not statistical significance. Growth-

restricted gilts at birth have delayed initiation of follicular development, with more primordial follicles, and fewer primary and secondary follicles compared to foetuses from gilts fed a diet meeting nutritional requirements for growth (Da Silva-Buttkus et al., 2003). Although not statistically significant, Da Silva-Buttkus et al. reported that there was a larger number of oogonial nests at birth in ovaries of foetuses from growth restricted animals. In the present study, there was a lesser number of primary follicles in the ovary of the foetuses of lesser weight compared with those with CTMLW at GD90, indicating there were differences in follicle number at this stage of gestation that were also present at the time of birth. Together, the histological and mRNA transcript abundance data warrant further examination into germ and somatic cell proliferation and degeneration throughout gestation, with a particular focus between days 45 and 90 of gestation.

Even though it is recognised that angiogenesis is essential for organ development and growth, and the processes of angiogenesis are integral for postnatal ovarian development, little is known regarding vascularisation of the foetal ovary. In the present study there was a decrease in the relative abundance of *CD31* mRNA transcript between GD60 and GD90. The CD31 protein is an endothelial cell marker, present at the

junctions between endothelial cells (Kim et al., 2013; Mamdouh et al., 2003). Furthermore, the CD31 protein is thought to be involved in angiogenesis by regulating endothelial cell migration (Cao et al., 2009, 2002; DeLisser et al., 1997; Matsumura et al., 1997; Yang et al., 1999; Zhou et al., 1999). Results from immunohistochemical evaluations for CD31 in the foetal ovaries at GD90 confirmed that CD31 is present in endothelial cells of the foetal ovary and there are indications that the foetal ovary has undergone marked vascularisation prior to this stage of development. It is hoped that further studies will be conducted to elucidate the timing and pattern of vascularisation in the foetal ovary.

The function of PGF2α in the development and maintenance of the corpora lutea (CL) is well characterised. There is no question as to whether PGF2α is essential for luteolysis and the establishment of pregnancy (Geisert and Bazer, 2015; McCracken et al., 1999; Moeljono et al., 1977; Przygrodzka et al., 2016, 2015; Waclawik et al., 2017; Ziecik et al., 2017). The function of the PTGFR in foetal ovarian development is not known. One hypothesis could be that the increase in abundance of PTGFR mRNA in the foetal ovary with advancing gestational day is indicative that there are ovarian developmental changes occurring in preparation

for postnatal ovarian functions. Binding of PGF2α to the PTGFR results in increased production of VEGFA by the endometrium, increasing angiogenesis (Kaczynski et al., 2016). Considering the functions of PGF2α in angiogenesis, it could also be hypothesized that this increase in abundance of PTGFR in late gestation may be a mechanism to further increase vascularization of the foetal ovary during this period of marked remodelling and growth. Because there was a decrease in the relative abundance of *PTGFR* mRNA transcript in the foetuses that were of a lesser weight compared to those of average weight at GD45 and GD60, this may warrant further investigation into angiogenesis in growth-restricted foetal ovaries during these stages of development. This study, to our knowledge, is the first in which there is evaluation of an association between foetal size and the relative abundance of *PTGFR* mRNA transcript in the foetal ovary of pigs. There should be further investigation of the protein to identify which cell types produce PTGFR and to elucidate the functions of PGF2α in gonadal development.

Growth restriction leads to a phenotype where pig foetuses are of a lesser weight, with impaired adaptation to extra-uterine conditions, lesser rates of pre-weaning survival, altered postnatal growth trajectories, undesirable carcass qualities and impaired reproductive performance

post-puberty (Wu et al., 2006). Male piglets are often considered to be at a disadvantage from a product productivity perspective postnatally (Baxter et al., 2012), and results from recent investigations assessing IUGR pigs have indicated female piglets are more likely to undergo compensatory growth than male IUGR piglets (Gonzalez-Bulnes et al., 2012). In the current study, several differences in gene expression, as evidenced by relative abundances of mRNA transcripts, were observed at GD45 or GD60 but not GD90. This could be interpreted either that there are changes that occur in the early foetal ovary which program the postnatal impaired reproductive phenotype in small gilts or that whilst there are marked changes in the foetal ovary early in development, there appears to be compensation in ovarian development as gestational stage advances.

5. Conclusions

These findings indicate there are differences in reproductive potential of lesser birthweight females postnatally that are programmed early in gestation. It is hoped that further investigation will improve the understanding of the association between prenatal reproductive development and postnatal reproductive performance.

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Author contribution statement

CS, CJA, and FXD devised the experiment. CS, CJA and COH collected samples. CS and YCA performed the experiments. CS, YCA and CJA analysed the data. COH provided technical support. CS and CJA prepared the final manuscript. All authors approved the manuscript.

Competing interest statement

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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Figure Legends

Fig. 1. Foetal ovarian weights and histological analyses at GD60 and GD90; Foetal paired ovarian weight (A) and paired ovarian weight as a percentage of body weight (B), number of oogonia (E), number of primordial follicles (F), number of primary follicles (G), and number of nuclei per oogonia (H) were compared between foetuses of different size; Mean values presented. *n* = 4-7 foetuses per group; Error bars represent S.E.M.; The association between foetal weight and paired ovarian weight at GD60 (C) and GD90 (D) was assessed.

Fig. 2. Maternal and foetal plasma oestradiol concentrations on GD90; Maternal plasma oestradiol concentrations were correlated with (A) number of live

930 foetuses and (B) percentage prenatal survival; Foetal
931 plasma oestradiol concentrations were compared between
932 foetuses of different size (C); Letters indicate that group
933 means differ from one another (P<0.05); n = 4-5 foetuses
934 per group; Error bars represent S.E.M.
935
936 **Fig. 3.** Candidate relative abundances of ovarian mRNA
937 transcripts in foetuses of different sizes at days 45, 60 and
938 90 of pregnancy; Relative abundance mRNA transcripts
939 for *BAX* (A), *BCL2* (B) *BAX:BCL2* Ratio (C), *CD31* (D),
940 *DMRT1* (E), *GATA4* (F), *HIF1A* (G), *INSL3* (H), *KI67* (I),
941 *P53* (J), *PTGFR* (K), *SPP1* (L) and *VEGFA* (M) in the
942 lesser weight to mean litter weight (CTMLW) foetal ovaries
943 at gestational days 45, 60 and 90; n = 3-6 foetuses per
944 group; Error bars represent S.E.M.; *P < 0.05.
945
946 **Supplementary Fig. 1.** Representative Images of GATA4
947 immunohistochemistry in foetal ovaries;
948 Immunohistochemistry results indicated that GATA4
949 protein is present in the somatic cells in both the foetuses
950 of lesser weight (D and F) and those closest to mean litter
951 weight (CTMLW) (C and E) at both gestational day (GD)
952 60 (C and D) and 90 (E and F); Rabbit IgG controls at an
953 equivalent protein concentration were utilised as a
954 negative control (A and B); Scale bars represent 100 μm.

955

956 **Supplementary Fig. 2.** Representative Images of CD31
957 Immunohistochemistry in foetal ovaries;
958 Immunohistochemistry confirmed that CD31 protein is
959 present in endothelial cells in the ovary of both the lesser
960 (C) and closest to mean litter weight (CTMLW) (B) foetuses
961 at gestational day (GD) 90; Rabbit IgG controls at an
962 equivalent protein concentration were utilised as a
963 negative control (A); Scale bars represent 100 μm.

964