Gestational nutrition 2: gene expression in sheep fetal ovaries exposed to gestational under nutrition

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Abstract

A number of studies have demonstrated effects of gestational undernutrition on fetal ovarian development and postnatal female fertility. However, the mechanism underlying these effects remains elusive. Using a cohort of animals in which altered gestational nutrition affected indicators of postnatal fertility, this study applies RNAseq to fetal ovaries to identify affected genes and pathways that may underlie the relationship between gestational plane of nutrition and postnatal fertility. Pregnant ewes were exposed to either a maintenance diet or 0.6 of maintenance for the first 55 days of gestation followed by an *ad libitum* diet. Complementary DNA libraries were constructed from 5 to 6 fetal ovaries from each nutritional group at both days 55 and 75 of gestation and sequenced using Ion Proton. Of approximately 16,000 transcripts, 69 genes were differentially expressed at day 55 and 145 genes differentially expressed at day 75. At both gestational ages, genes expressed preferentially in germ cells were common among the differentially expressed genes. Enriched gene ontology terms included ion transport, nucleic acid binding, protease inhibitor activity and carrier proteins of the albumin family. Affected pathways identified by IPA analysis included LXR/RXR activation, FXR/RXR activation, pathways associated with nitric oxide production and citrullination (by *NOS1*), vitamin C transport and metabolism and REDOX reactions. The data offer some insights into potential mechanisms underlying the relationship between gestational plane of nutrition and postnatal fertility observed in these animals. In particular, the roles of nitric oxide and protease inhibitors in germ cell development are highlighted and warrant further study.

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Introduction

The effects of gestational nutrition on fetal ovarian development and subsequently the effects on fertility in female offspring have been studied in a range of species, with sheep being a popular model for these studies. Previous studies in sheep have shown restricted gestational nutrition affects fetal ovarian development by decreasing fetal ovarian weight (Rae *et al.* 2001, Grazul-Bilska *et al.* 2009), decreasing germ cell proliferation (Lea *et al.* 2006) and germ cell number (Rae *et al.* 2001), increasing primordial follicle number and decreasing numbers of growing follicles (Rae *et al.* 2001). Conversely, overfeeding sheep during gestation has been shown to affect fetal ovarian development by increasing follicle numbers (Da Silva-Buttkus *et al.* 2003, Asmad *et al.* 2015) and increasing germ cell numbers (Borwick *et al.* 1997). Regardless of whether gestational plane of nutrition was above or below maintenance levels, impacts on the ovaries of female offspring have been reported (Rae *et al.* 2002, Kotsampasi *et al.* 2009, Long *et al.* 2010). These impacts had predominantly negative effects on fertility

of the offspring (Gunn *et al.* 1995, Rae *et al.* 2002, Long *et al.* 2010).

The mechanism(s) underlying the effects of gestational nutrition on fetal ovarian development and/or adult female fertility are yet to be established. Potential mechanisms including nutrient delivery, endocrine status, changes to the reproductive axis and gene expression are broadly outlined by Rhind *et al.* (2004). However, given the relationship between establishment of the ovarian reserve, antral follicle count and fertility (Broekmans *et al.* 2006, Ireland *et al.* 2008, 2010), it seems logical that potential mechanisms during ovarian development are likely to target germ cell development. Increased germ cell DNA damage has been reported in fetal ovaries exposed to restricted gestational nutrition (Murdoch *et al.* 2003) and offers one mechanism by which restricted gestational nutrition may affect fetal ovarian development and subsequently the fertility of female offspring.

There is a paucity of data examining how changes in maternal nutrition may affect the fetal ovarian transcriptome and the implications of altered gene expression on ovarian development. While some studies

have focused on key genes involved in development (Da Silva *et al.* 2003), few have examined the whole transcriptome. Costa used RNAseq to examine changes in the fetal ovarian transcriptome at day 60 of gestation from cows on a restricted nutrition regime. Differential expression of 79 genes (from a total of 20,000) was noted with some of these genes involved in embryonic or ovarian development (Costa *et al.* 2014). Lea used microarray to examine gene expression in fetal ovaries where maternal ewes were exposed to sewage sludge for a range of periods during gestation (Lea *et al.* 2016). Between 4 and 120 transcripts were differentially expressed depending on the exposure time. Differentially expressed transcripts were involved in functions such as cell growth and differentiation, cell cycle regulation, cell death, cell differentiation and cell movement.

In a companion paper (ref companion paper), restricted gestational nutrition in sheep from day 0 to 55, followed by *ad libitum* feeding, resulted in alterations to fetal ovarian development, and surprisingly, increased indicators of fertility in female offspring. To examine potential mechanisms underlying the effects of gestational nutrition in this model, RNAseq was performed to examine gene expression patterns in fetal ovaries from the same cohort of animals described in the companion paper.

Materials and methods

Animal model

All animal manipulations were carried out in accordance with the 1999 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand and were approved by the Invermay Agricultural Centre Animal Ethics Committee (AE13294, 13098 and 12820).

Details of animals and diets have been described previously (ref companion paper). Briefly, on the second cycle following synchronization, 79 five-year-old Romney $\times$ Coopworth ewes were mated to intact Texel rams. From the day of mating, ewes were housed indoors with 39 ewes fed a maintenance diet (comprising sheep pellets, chaff and silage) and 40 ewes were fed a diet equivalent to 0.6 of maintenance based on metabolizable energy intake. For maintenance ewes, this was calculated at 14.5 megajoules of metabolizable energy per day (using the formula $0.6 \times \text{ewe weight (kg)}^{0.75}$, supplied by the AgResearch Small Ruminant Research Unit, Palmerston North, NZ). Those ewes on 0.6 maintenance diet received 8.7 megajoules per day. At day 55 of gestation, ewes were then fed *ad libitum* pasture. A subgroup of these ewes ($n=23$) were assigned for fetal collections. Following a barbiturate overdose, female fetuses were recovered at day 55 and 75. The right ovaries of fetuses from unique dams were weighed, snap frozen in liquid nitrogen and stored at -70°C until required for RNA extraction ($n=6$ maintenance and six restricted at day 55 and $n=6$ maintenance and five restricted at day 75).

RNA extraction

RNA extractions were performed using the Zymo duet kit (Zymo Research Corporation, Irving, CA, USA. Cat D7001). Fetal ovaries were homogenized in 800 μL of lysis buffer for 30s (Ultraturrex T25, Ika Labortechnik, Germany), with the homogenate then split into two equal aliquots. Each aliquot was centrifuged at 12,000 g for 1 min through 2 DNA binding columns followed by centrifugation (12,000 g for 1 min) through an RNA-binding column. Prescribed washing steps were incorporated in accordance with the manufacturer's instructions, and an in-column DNase digest performed following the manufacturer's instructions. The RNA was eluted from each column by centrifugation (10,000 g for 30s) with 55 μL of DNase free water. To increase RNA recoveries, the eluate was reapplied to the column and the centrifugation was repeated. A further-off column DNase digest was performed using the Ambion DNA-free kit following the manufacturer's guidelines (Ambion). Samples were stored at -70°C .

RNA quantity and quality were measured using a Bioanalyser 2100 and the RNA 6000 pico kit (Agilent Technologies). Potential DNA contamination of RNA samples was determined using a Qubit assay (dsDNA BR, Thermo Fisher Scientific) following the manufacturer's instructions.

mRNA enrichment

ERCC control mixes (Ambion ERCC Spike-In Control Mixes, Cat 4456739. Thermo Fisher Scientific) were added to total RNA samples (6 μL of the supplied reagent at a 1:100 dilution in nuclease free water to 6 μg of total RNA) prior to mRNA enrichment. The RNA aliquots (each containing 6 μg of total RNA) were diluted to 150 μL with nuclease free water and mRNA enrichment undertaken using the Dynabead mRNA Direct Micro kit (Life Technologies), following the manufacturer's instructions for samples containing 1–50 μg of total RNA.

Library preparation

Whole transcriptome libraries were constructed using the Ion Total RNAseq kit V2 (Thermo Fisher Scientific) as per the manufacturer's instructions for fragmented poly (A) RNA up to 5 μg . A 3 μg aliquot of each mRNA enriched sample was used for library construction. Initial fragmentation using the supplied RNaseIII was for 3 min at 37°C . Library-specific barcodes were ligated to each library made from an individual ovary. Library quality and quantity was assessed using a Bioanalyser 2100 and the DNA 1000 pico kit (Agilent Technologies).

Sequencing

The emulsion PCR and enrichment process was performed using the Ion One Touch 2 System and the Ion PI Template OT2 200 Kit v3 (Thermo Fisher Scientific), following the manufacturer's instructions. Each emulsion PCR and sequencing process used a total of 100 μL of pooled library (comprising a pool of between 3 and 12 barcoded libraries) at a final concentration of 10 pmol/L of cDNA.

Ion Proton sequencing was performed using Ion PI v2 chips. To control for technical variation, a total of 15 chips were sequenced, with each chip containing between 3 and 12 libraries. Each barcoded library was sequenced on 3–5 chips.

Data processing

The Fastx Tool Kit (v 0.0.14, http://hannonlab.cshl.edu/fastx_toolkit/) was used to filter and trim low-quality sequences (>40% of bases with a Phred score <25), remove low-quality (Phred score <25) bases from the 3' end of sequencing reads and discard sequences shorter than 25 bases. Sequence quality was assessed using FastQC (v0.10.1).

Raw sequencing reads were mapped to a reference genome, *Ovis Aries* 3 (OAR3.1, GCF_000298735.1), (Archibald *et al.* 2010, Jiang *et al.* 2014) using STAR (v2.3.0.1) (Dobin *et al.* 2013). Data for each ERCC transcript (length and sequence) were incorporated into the sheep genome reference files. Sequencing files for each ovarian library were converted and merged to produce a single SAM file for each ovary using Samtools (v0.1.19-44428) (Li *et al.* 2009). Files were analyzed using Cufflinks (v2.2.1) (Trapnell *et al.* 2012) to generate expressed gene lists. Differential gene expression analysis was performed using Cuffdiff (v2.2) (Trapnell *et al.* 2012). The Benjamini–Hochberg procedure was applied to correct for multiple comparisons. A false discovery rate of $q < 0.05$ was used as cut-off criteria to assign significance. Significant positive fold changes ranged from 1.4 to 58.3 and significant negative fold changes ranged from 1.1 to 20.0. At each gestational age, fragments per kilobase of exon per million reads mapped (fpkm) values for ovaries from the restricted group are compared to the fpkm values for the maintenance group; thus, a positive fold-change indicates upregulation in the restricted group compared to the maintenance group, while a negative fold change indicates downregulation in the restricted group.

To determine potential biological pathways affected, and the functional significance of genes with significant differential expression (SDE) both Ingenuity Pathway Analysis (IPA, Qiagen) and Gene Ontology terms (GO terms) sourced from AMIGO (Carbon *et al.* 2009) were used. GO terms were analyzed for enriched terms using the Batch Genes tool and default settings in GOEAST (Zheng & Wang 2008).

Determination of the lower limit of detection (LLD) from ERCC control mixes

The average measured fpkm value for each detected ERCC transcript was plotted against the known concentration of that transcript in the ERCC standard. Outliers were removed from the analysis until an R^2 value >0.9 was achieved. To achieve an R^2 value >0.9, all fpkm values <0.1 were removed. One additional transcript (ERCC0074) was also removed. ERCC0074 returned an average fpkm value of 1500, almost three-fold higher than any sample value in the dataset. The resulting linear relationship between known and measured concentrations indicated that the LLD for this dataset was 0.1 fpkm. No fpkm values from the dataset were greater than the upper most remaining ERCC standard.

Validation of fold changes using Nanostring

The Nanostring nCounter XT gene expression assay (Nanostring Technologies, Seattle, WA, USA) was used to compare fold changes for a range of genes. The manufacturer's protocol was followed using 4 μ L of total RNA from the same extract used for RNAseq. Counts were normalized to housekeeping genes where RNAseq results showed consistent expression levels over both groups and both ages (*YWHAZ*, *SDHA*, *GAPDH* and *G6PD*) and analysis performed using the Nanostring N solver software (v2.6). Based on RNAseq gene expression data, genes with a range of fold-changes, a range of significance levels between groups, and a range of expression levels, were selected for Nanostring analysis. A total of 50 genes were selected that included the housekeeping genes. Code sets (reporter and capture probes) were synthesized by Nanostring Technologies (Nanostring Technologies).

Results

Library and sequencing metrics

Average RNA and library concentrations are presented in Table 1 for all 23 samples used for library construction. The average RIN value for RNA samples was 9.1 (range 8.8–9.8) and DNA contamination was below the detection limit of the assay employed (10 ng/ μ L). Library fragment lengths were <200bp.

Following sequencing, each library produced an average of 37.3 million reads, with an average read length of 84bp, a GC content of 49%, and a Phred score of 24. Following trimming and filtering, read length was reduced to 69bp, and Phred score increased to 25. The average per base mismatch rate over all libraries was 0.18%. Seventy one percent of reads aligned to the sheep genome resulting in an average of 18.6 million aligned reads per library.

Overall gene expression

At day 55, 14,384 (maintenance) and 14,530 (restricted) annotated genes returned expression values greater than the LLD (0.1 fpkm), while 3406 (maintenance) and 3788 (restricted) unannotated genes returned expression values greater than the LLD. At day 75, 15,038 (maintenance) and 15,009 (restricted) annotated genes returned expression values greater than the LLD (0.1 fpkm), while 3567 (maintenance) and 3562 (restricted) unannotated genes returned expression values greater than the LLD.

Differential gene expression

Complete lists of SDE genes at days 55 and 75 are available in Supplementary data 1 (see section on supplementary data given at the end of this article). The numbers of SDE genes are given as metrics in Table 2 and as volcano plots in Fig. 1. While at day 75 over

Table 1 RNA recoveries and cDNA library concentrations.

	Day 55 maintenance	Day 55 restricted	Day 75 maintenance	Day 75 restricted
Ovary weight (mg)	10.3±0.9	10.1±0.7	23.9±1.3	24.9±1.3
RNA concentration (ng/μL)	104.5±13.5	135.4±18.7	332±14.8	302.1±35.9
Library concentration (ng/μL)	13.8±1.9	13.5±2.0	14.5±2.4	18.3±1.9
Library molarity (nmol/L)	130.0±18.8	135.1±17.1	119.2±17.9	165.3±12.0

twice as many genes were differentially expressed, the fold changes were considerably less than those observed at day 55. The average negative fold change at day 75 was -2.0 (this excludes *CCL16*, this gene, involved in immunoregulatory and inflammatory responses, was expressed in maintenance ovaries but no expression was evident in restricted ovaries), while at day 55 this was -9.0 . The average positive fold change at day 75 was 1.8 while at day 55 this was 5.0 .

The ten most SDE genes (by both positive and negative fold change) are presented in Table 3 (day 55) and Table 4 (day 75). Cell-specific expression patterns of these genes within the fetal ovary have been sourced from a number of studies over a variety of mammalian species. These studies were selected so that the developmental timing was similar to the current study (Choi *et al.* 2007, Fowler *et al.* 2009, Regassa *et al.* 2011, Zhang *et al.* 2011, Jameson *et al.* 2012, Bellingham *et al.* 2013, Bonnet *et al.* 2013, Li *et al.* 2013, Bebbere *et al.* 2014, Soh *et al.* 2015). GO annotations were sourced from (Carbon *et al.* 2009). Of the 55 known SDE genes identified at day 55, the cell-specific expression pattern of 35 genes has been published. Of these 35 genes, 16 are expressed preferentially or exclusively in germ cells. Four of these genes, while their expression pattern in fetal ovaries is unknown, are reported to be expressed specifically in adult oocytes, a further 13 are expressed in all ovarian cell types including germ cells, and two have been shown not to be expressed in germ cells. Of the 122 SDE genes at day 75, 25 are known to be specifically or preferentially expressed in germ cells, 11 while their expression pattern in fetal ovaries is unknown, are known to be specifically or preferentially expressed in adult oocytes, 13 are known to either not be expressed in germ cells or have low expression in germ cells and 34 are known to be expressed in all cells of the fetal ovary including germ cells. The cell-specific expression of 39 genes is unknown.

Of the most upregulated genes in restricted ovaries at day 55, the GO annotations can largely be assigned to two categories: nucleic acid binding and ion transport (Table 3). Genes downregulated in restricted ovaries can be assigned to only two GO categories: carrier proteins of the albumin family and those with protease inhibitor activity. While GO annotations for *VTN* do not include protease inhibitor activity, *VTN* is known to bind to several protease inhibitors (Deng *et al.* 1996) and has been shown to play a role in proteolytic cascades (Preissner *et al.* 1997). While less clear cut, this pattern

of GO annotations remains present for the most SDE genes at day 75 (Table 4).

Affected pathways presented are based on the *P* value generated by IPA, indicating that the expression of a significant number of genes within that pathway was affected by the nutritional regime. While *z* scores are used by IPA to indicate activation or inhibition or pathways, in this dataset *z* scores could not be calculated for most of the affected pathways. Of the 15 pathways presented at day 55 (Table 5), *z* scores were calculated for only two pathways, there was insufficient data (<four target genes affected) for three pathways, while the remaining ten pathways were ineligible for activity analysis due to insufficient information in the literature of QIAGEN Knowledge Base. At day 75, of the 21 pathways presented (Table 6), *z* scores were generated for three pathways, there was insufficient data for four pathways and the remaining 14 pathways were ineligible for activity analysis. The top canonical pathways identified by IPA as affected at day 55 (Table 5) included LXR/RXR activation, FXR/RXR activation, acute phase response signaling, protein citrullination, citrulline-nitric oxide cycle, nNOS signaling in skeletal muscle, super pathway of citrulline metabolism and calcium transport 1. Of these, LXR/RXR activation was predicted as downregulated while for the remainder low activation scores (based on the expression levels/patterns of key focus genes within a pathway) meant activity patterns were not able to be predicted. At day 75, canonical pathways affected (Table 6) included acute phase response signaling, nitric oxide signaling in skeletal muscle, LXR/RXR activation, FXR/RXR activation, production of nitric oxide and reactive oxygen species in macrophages, vitamin C transport, ascorbate recycling, glutathione redox reactions, citrulline-nitric oxide cycle and protein citrullination. Of these, LXR/RXR activation, and production of nitric oxide (NO) and reactive oxygen species (ROS) in macrophages were

Table 2 Summary of differential gene expression between fetal ovaries whose dams were exposed to either maintenance or restricted nutrition.

Differentially expressed sequences	Day 55	Day 75
Total	69	145
Unassigned to a gene identifier	14	23
Known genes common to both ages	16	
Known genes unique to each age	39	106
% Upregulated in restricted group	51	66

Figure 1 Volcano plots illustrating fold change vs false discovery rate. (A) Day 55 genes and (B) day 75 genes. Differentially expressed genes (between nutritional groups) are represented by red dots. Genes not differentially expressed are represented by black dots.

predicted as downregulated while for the remainder the activity pattern could not be predicted.

Validation of changes using Nanostring

Of the 46 genes analyzed by Nanostring, 18 returned at least one sample which was below the detection limit of the Nanostring nCounter XT gene expression assay. These genes were removed from the comparative analysis. The remaining data are presented as a Bland–Altman plot (Fig. 2) comparing Nanostring and RNAseq fold changes. An R^2 value of 0.92 indicates a strong, consistent proportional error situation. That is, the larger the fold change then the larger the difference between the two techniques, where the fold change detected by Nanostring is larger than that detected by RNAseq.

However, in the context of this study, the high R^2 value demonstrates a consistency between the techniques and that Nanostring data support the scale and direction of the fold changes obtained using RNAseq. Fold changes for RNAseq and Nanostring for each gene at each age are presented in [Supplementary data 2](#).

Discussion

The companion paper (ref companion paper) reported increased indicators of fertility in female offspring from dams exposed to an initial restricted plane of nutrition. Further, while no differences were observed in the pattern of fetal germ cell development at the cessation of the nutrition restriction period (day 55), Twenty days following the change to *ad libitum* feeding, fetal ovaries from offspring of dams exposed to restricted nutrition contained more germ cells but a lower proliferation rate was apparent. While the area staining positive for autophagy (MAPLC3B) was not different between nutritional groups, the intensity of this staining was higher in day 75 fetal ovaries from dams exposed to restricted nutrition. No differences were apparent between nutrition groups in the levels of germ cell apoptosis at both day 55 and 75 of gestation (as determined by TUNEL staining). The appearance of most changes in morphological aspects of germ cell development only after the transition from a restricted plane of nutrition to *ad libitum* nutrition suggests that it is the change in diet which may be important in establishing the observed effects. The objective of the current study was to examine fetal ovaries, from the same cohort of animals,

Table 3 Most differentially regulated genes (by fold change) at day 55 of gestation.

Gene	Reported expression	Maintenance fpkm	Restricted fpkm	GO annotation(s)
ZP3	Germ cell	0.1 ± 0.03	7.3 ± 7.0	Ion transport
FOXR1	Germ cell	0.3 ± 0.04	5.5 ± 5.0	Transcription factor, nucleic acid binding
FIGLA	Germ cell	0.8 ± 0.3	8.2 ± 7.4	Transcription factor, nucleic acid binding
PADI6	Germ cell	2.5 ± 0.2	15.9 ± 13.0	Ion transport
KIAA1324L	Unknown	0.5 ± 0.1	2.8 ± 2.3	Ion transport
HIST2H2BF	Unknown	5.3 ± 1.2	29.9 ± 23.0	DNA binding
GDF9	Germ cell	2.5 ± 3.2	9.4 ± 6.7	Cytokine activity
NOS1*	Germ cell	0.5 ± 0.01	1.7 ± 0.9	Ion transport, protein binding
PNLDC1	Germ cell	2.5 ± 0.5	7.8 ± 6.1	Nucleic acid binding
SAMD15	Unknown	2.4 ± 0.6	6.9 ± 4.7	Unknown function
AFP	Unknown	30.0 ± 19.5	0.6 ± 0.2	Carrier protein albumin family
FETUB	Germ cell	10.7 ± 6.4	0.3 ± 0.1	Protease inhibitor
SERPINA1	Unknown	84.8 ± 50.2	3.3 ± 1.3	Protease inhibitor
AMBP	Unknown	7.2 ± 4.0	0.3 ± 0.1	Protease inhibitor vesicle development
GC	All	5.3 ± 3.7	0.2 ± 0.1	Carrier protein albumin family
AHSG	Unknown	184.8 ± 105.2	10.5 ± 8.3	Protease inhibitor vesicle development
ALB	Germ cell	16.1 ± 9.5	1.3 ± 0.2	Carrier protein albumin family
VTN	Not germ cell	5.6 ± 3.7	0.5 ± 0.4	Binds to protease inhibitors
SPP2	Unknown	6.4 ± 4.0	0.6 ± 0.3	Protease inhibitor
ITIH2	Unknown	2.2 ± 1.4	0.2 ± 0.2	Protease inhibitor

Positive fold change indicates upregulation in restricted ovaries. Expression patterns are sourced from (Choi et al. 2007, Fowler et al. 2009, Regassa et al. 2011, Zhang et al. 2011, Jameson et al. 2012, Bellingham et al. 2013, Bonnet et al. 2013, Li et al. 2013, Bebbere et al. 2014, Soh et al. 2015).

*Indicates adjusted P value < 0.05, all other q values < 0.01.

Table 4 Most differentially regulated genes (by fold change) at day 75 of gestation.

Gene	Expression	Maintenance fpkm	Restricted fpkm	GO annotation(s)
<i>SLC12A3*</i>	Oocyte (adult)	1.2 ± 0.2	3.4 ± 1.3	Ion transport
<i>ADAMTS14</i>	Oocyte (adult)	5.3 ± 0.7	13.4 ± 3.4	Peptidase activity
<i>TDRD10</i>	Germ cell	21.8 ± 1.3	49.9 ± 11.6	Nucleic acid binding
<i>MATER</i>	Germ cell	3.1 ± 0.5	7.1 ± 2.2	Germ cell development
<i>RBM20</i>	Unknown	1.7 ± 0.1	3.8 ± 0.4	Nucleic acid binding
<i>CHL1</i>	Oocyte (adult)	9.7 ± 2.1	21.8 ± 8.4	Protease binding
<i>SPTBN5</i>	Unknown	1.1 ± 0.1	2.4 ± 0.5	Actin binding
<i>NLRC5</i>	All cells	2.2 ± 1.0	4.7 ± 1.0	DNA binding
<i>TSKS*</i>	Germ cell	4.3 ± 0.7	9.4 ± 1.2	Protein kinase binding
<i>RYR3</i>	Unknown	0.9 ± 0.1	2.0 ± 0.4	Ion transport
<i>CCL16#</i>	Unknown	2.3 ± 1.0	0.0 ± 0.0	Chemokine activity
<i>AMBP</i>	Unknown	9.0 ± 2.2	1.3 ± 1.2	Protease inhibitor
<i>AHSG</i>	Unknown	108.9 ± 28.2	18.3 ± 15.9	Protease inhibitor
<i>PLG</i>	Unknown	1.8 ± 0.5	0.4 ± 0.2	Protease inhibitor
<i>SERPINA1</i>	Oocyte (adult)	57.4 ± 17.1	13.4 ± 0.5	Protease inhibitor
<i>AFP</i>	Unknown	12.5 ± 4.6	2.9 ± 2.7	Carrier protein albumin family
<i>ALB</i>	Unknown	16.3 ± 4.7	4.1 ± 3.0	Carrier protein albumin family
<i>APCDD1L</i>	Unknown	21.2 ± 5.9	7.1 ± 1.2	Membrane component
<i>MOXD1</i>	Unknown	15.7 ± 2.5	6.7 ± 0.8	Ion transport
<i>CALB2</i>	Unknown	18.3 ± 3.9	9.0 ± 1.6	Ca transport

Positive fold change indicates upregulation in restricted ovaries. Cell-specific expression pattern GO annotations are sourced as described for Table 3.

#Represents expression in maintenance ovaries and no expression in restricted ovaries. *Indicates adjusted *P* value <0.05, all other *q* values <0.01.

using RNAseq, to identify genes and potential pathways affected by differences in maternal plane of nutrition.

At day 55 (the cessation of nutrition restriction), 69 genes were differentially expressed while at day 75 (nutrition restriction followed by 20 days *ad libitum* feeding), 145 genes were differentially expressed. The number of differentially expressed genes are consistent with the findings of both Costa *et al.* (2014) and Lea *et al.* (2016). Subsequent analysis identified a number of pathways and GO terms linking many of

these differentially expressed genes. The potential for differential expression to affect germ cell development is highlighted when examining the most affected genes by fold change at each age. Of those genes with a known cell-specific expression pattern, 9/11 at day 55 and 3/7 at day 75 are expressed either exclusively or preferentially in germ cells, the remainder is expressed in all cell types or their expression pattern in the fetal ovary is unknown.

The focus of this discussion is exploring how the affected genes, pathways and GO terms may contribute

Table 5 Day 55 most affected canonical pathways identified by IPA.

Ingenuity canonical pathways	<i>P</i> value	Molecules
Acute phase response signaling**	1.23E-08	AGT↓, APOA2↓, FGB↓, APOH↓, SERPINA1↓, AHSG↓, AMBP↓, ITIH2↓, ALB↓
LXR/RXR activation**	1.55E-08	AGT↓, VTN↓, APOA2↓, APOH↓, SERPINA1↓, AHSG↓, AMBP↓, ALB↓
FXR/RXR activation	2.14E-08	AGT↓, VTN↓, APOA2↓, APOH↓, SERPINA1↓, AHSG↓, AMBP↓, ALB↓
Embryonic stem cell differentiation into cardiac lineages	5.89E-04	NANOG↓, POU5F1↓
Transcriptional regulatory network in embryonic stem cells	9.55E-03	NANOG↓, POU5F1↓
Role of Oct4 in mammalian embryonic stem cell pluripotency*	1.26E-02	NANOG↓, POU5F1↓
Myo-inositol biosynthesis	1.48E-02	IMPAD1↓
Protein citrullination	1.82E-02	PADI6↑
Citrulline-nitric oxide cycle	1.82E-02	NOS1↑
NRF2-mediated oxidative stress response*	2.88E-02	ACTB↑, GSTM3↑, DNAJB14↓
Production of nitric oxide and ROS in macrophages*	2.88E-02	APOA2↓, SERPINA1↓, ALB↓
Calcium transport I	3.63E-02	ATP2C2↓
Mouse embryonic stem cell pluripotency	4.79E-02	NANOG↓, POU5F1↓
Super pathway of citrulline metabolism	5.01E-02	NOS1↑

Arrows indicate up or down-regulation in restricted fetal ovaries. All remaining pathways were not eligible for predictions of activation or inhibition because there is not enough information in the literature of Qiagen Knowledge Base at this time about how expression patterns of molecules affect their activity.

*Indicates there is not enough evidence from the dataset to make a prediction of activity; **z score calculated at -2.8 for LXR/RXR activation and zero for acute phase response signaling.

Table 6 Day 75 most affected canonical pathways identified by IPA.

Ingenuity canonical pathways	P value	Molecules
Acute phase response signaling**	2.69E-03	SERPINA1↓, AHSG↓, AMBP↓, PLG↓, ALB↓
nNOS signaling in skeletal muscle cells	3.16E-03	RXR3↑, NOS1↑
Vitamin C transport	3.16E-03	GLRX↑, SLC2A1↓
Production of nitric oxide and ROS in macrophages**	3.47E-03	IRF8↑, JAK3↓, SERPINA1↓, MAP3K15↑, ALB↓
LXR/RXR activation**	4.79E-03	SERPINA1↓, AHSG↓, AMBP↓, ALB↓
FXR/RXR activation	5.62E-03	SERPINA1↓, AHSG↓, AMBP↓, ALB↓
Cardiolipin biosynthesis II	1.12E-02	PGS1↓
Inhibition of angiogenesis by TSP1*	1.55E-02	HSPG2↓, GUCY1A3↑
Coagulation system*	1.66E-02	SERPINA1↓, PLG↓
Biotin-carboxyl carrier protein assembly	1.66E-02	ACACB↑
Tyrosine biosynthesis IV	1.66E-02	PCBD1↑
Ascorbate recycling (cytosolic)	2.24E-02	GLRX↑
Glutathione redox reactions II	2.24E-02	GLRX↑
Phenylalanine degradation I (aerobic)	2.24E-02	PCBD1↑
Corticotropin releasing hormone signaling	2.45E-02	NOS1↑, CNR1↓, GUCY1A3↑
Protein citrullination	2.75E-02	PADI6↑
Citrulline-nitric oxide cycle	2.75E-02	NOS1↑
Pentose phosphate pathway (non-oxidative branch)	3.31E-02	TKT↓
IL-12 signaling and production in macrophages	3.89E-02	IRF8↑, SERPINA1↓, ALB↓
Synaptic long term depression*	4.57E-02	RXR3↑, NOS1↑, GUCY1A3↑
Antiproliferative role of somatostatin receptor 2*	4.90E-02	NOS1↑, GUCY1A3↑

Arrows indicate up or down-regulation in restricted fetal ovaries. All remaining pathways were not eligible for predictions of activation or inhibition because there is not enough information in the literature of QIAGEN Knowledge Base at this time about how expression patterns of molecules affect their activity.

*Indicates there is not enough evidence from the dataset to make a prediction of activity. **z score calculated at -2.0 for LXR/RXR Activation, 0.45 for production of nitric oxide and ROS in macrophages and zero for acute phase response signaling.

to the phenotypic effects observed in both the day 75 fetal ovaries, and the female offspring from this cohort of animals reported in the companion paper.

The oocyte-specific transcription factor *FIGLA* showed a ten-fold upregulation at day 55 ($q=0.01$) and a two-fold upregulation at day 75 ($q=0.02$). *FIGLA* is known to regulate expression of the zona pellucida (ZP) genes (Joshi et al. 2007). Of the ZP genes, there was a 58 fold upregulation of *ZP3* at day 55 ($q<0.01$), while large fold changes were observed for both *ZP2* and *ZP4* at this age, these were not significant due to large variation between individual ovaries ($q=0.3$ and 0.9 respectively). Electron microscope examination of sheep fetal ovaries at day 55 of gestation did not reveal indications of ZP formation (Sawyer et al. 2002). Similarly, expression of *ZP3* has also been observed in human ovaries prior to follicle formation (Törmälä et al. 2008). Thus, it appears likely that at this stage of development, *ZP3* is playing some alternative role in germ cell development rather than zona pellucida formation. GO annotations for *ZP3* include both signal transducer activity and Ca^{2+} channel activity.

The role of *ZP3* in Ca^{2+} channel activity is of particular interest as IPA identified ion transport as a pathway affected by nutritional restriction. Further, at least three other upregulated germ cell specific/preferential genes have GO annotations relating to ion transport and binding: *NOS1* (3.4 fold at day 55, $q=0.03$; 2 fold at day 75, $q=0.01$), *PADI6* (6.4 fold at day 55, $q=0.01$; 2.1 fold at day 75, $q=0.01$) and

KIAA1324L (6 fold at day 55, $q=0.01$). Ca^{2+} is known to play key roles in other affected pathways particularly citrullination and nitric oxide production by *NOS1* (Clementi 1998). Conversion of arginine to nitric oxide and citrulline by *NOS1* is a Ca^{2+} -dependent reaction (Wendehenne et al. 2001), this reaction is also catalyzed by Ca^{2+} dependent peptidylarginine deaminases including *PADI6* (Mohamed et al. 2012). *NOS1* and *PADI6* are upregulated in fetal ovaries from dams exposed to restricted nutrition at both day 55 (3.4-fold and 6.4-fold respectively) and day 75 (2-fold and 2.1-fold respectively).

Figure 2 Bland–Altman plot comparing fold changes obtained from Nanostring and RNAseq for 28 genes. Vertical axis represent mean fold change of the two techniques, horizontal axis represents the difference in fold change obtained from the two methodologies.

NO is known to play a role in DNA damage and repair although its precise role is complex with NO-induced DNA damage occurring through a variety of cell-specific and/or dose-dependent mechanisms as described by Tamir *et al.* (1996). However, it is primarily not NO *per se*, which causes DNA damage, but the products (such as N_2O_3 and ONOO) of the reaction between NO and ROS (Burney *et al.* 1999). As *NOS1* and *PADI6* are involved in NO production, are preferentially expressed in germ cells and are upregulated in nutritionally restricted fetal ovaries at a time of significant germ cell proliferation, apoptosis and meiosis, it seems logical to hypothesize that this upregulation may have a negative effect on the DNA status in these cells. Supporting this contention, Murdoch *et al.* demonstrated in sheep, increased oxidative base lesions in DNA of day 78 fetal oogonia exposed to restricted nutrition from day 28 of gestation (Murdoch *et al.* 2003). While Murdoch did not examine the mechanism by which the increased lesions arise, we hypothesize that in a restricted maternal nutritional environment NO may be a contributing factor.

The improved indicators of fertility in the offspring from this cohort of animals described in the companion study would suggest however beneficial effects on germ cell development. The companion study also highlighted the potential importance of the change in diet from restricted to *ad libitum*. In the current study, at day 75 following the change to *ad libitum* feeding, IPA identified three affected pathways –glutathione REDOX reactions, vitamin C transport and ascorbate recycling, which may have beneficial effects by blocking NO-induced DNA damage during the crucial period where meiosis is prevalent (day 55–75).

One of the major functions of the glutathione REDOX pathway is to regulate the intracellular levels of ROS (Mailloux *et al.* 2013). Vitamin C (ascorbate) is one compound that can potentiate NO synthesis (Heller *et al.* 2001) and is also a known antioxidant. Treatment with vitamin C has been shown to inhibit ROS generation and afford protection to spermatozoa in rats (Hsu *et al.* 1998), and the potential for vitamin C to afford some protection from DNA damage particularly in an under-nourished situation has been reported previously (Sram *et al.* 2012). Additionally, Zhang reported that fetal germ cells from mice deficient in *NOS3*, and exposed to the environmental estrogen bisphenol A, showed an increase in chromosomal errors during meiosis (Zhang 2007), suggesting a protective effect of NO in fetal germ cells. Thus, these three affected pathways have the potential to limit NO-induced DNA damage and promote the beneficial effects of NO on cell survival, proliferation and apoptosis as described by Napoli *et al.* (2013). Potentially further contributing to an improved germ cell DNA status at day 75 are two upregulated genes involved in DNA repair, *LIG1* (1.5 fold, $q=0.03$) and *PARP2* (1.5 fold, $q=0.03$). Potentially further supporting this concept is the finding at day 75

of expression of *CCL16* in maintenance ovaries while in restricted ovaries there was no detectable expression of *CCL16*. Expression of *CCL16* has been shown to increase in response to oxidative stress (Shini & Kaiser 2009), thus, the lack of expression in restricted ovaries at day 75 is consistent with the concept of lower oxidative DNA damage in these ovaries at day 75.

The role of NO in ovarian development is complex as NO is involved in numerous cell and developmental processes. Additionally, *NOS* is present in three distinct isoforms (Wendehenne *et al.* 2001). The potential importance of the *NOS* and citrullination pathways in fetal development has been highlighted by Wu *et al.* (2004) although largely in the context of placental development and/or angiogenesis. In sheep, allantoic fluid concentrations of citrulline increase 18-fold between days 30 and 60 (Kwon *et al.* 2003). Maternal nutritional restriction decreases fetal plasma arginine concentrations in the pig at day 60 of gestation (Wu *et al.* 1998). Restricted maternal nutrition in sheep between days 28 and 78 of gestation decreases maternal and fetal plasma levels of both arginine and citrulline (Kwon *et al.* 2004). A study using *NOS3*-deficient mice showed a relationship between impaired NO production and intra-uterine growth retardation (Heffler *et al.* 2001), a relationship that can be reversed by arginine supplementation (Vosatka *et al.* 1998). While there is no evidence to suggest that fetal ovarian *NOS1* (or *PADI6*) contribute to these observations, it does however highlight the potential significance of the arginine-citrulline-NO pathways in fetal development and provides some evidence that these pathways can be influenced by maternal nutrition.

NO is also known to play an important role in angiogenesis, although this function is normally attributed to *NOS3* (Cooke 2003), which in the fetal ovary is predominantly expressed in endothelial cells (Jameson *et al.* 2012) and not differentially expressed between nutritional groups. As *NOS1* and *PADI6* expression is localized to either germ cells or pregranulosa cells, it seems unlikely that the differences in *NOS1* and *PADI6* expression would have a significant effect on angiogenesis.

The discussion of potentially elevated levels of germ cell NO (via *NOS1*) may seem at odds with the predicted downregulation of the production of NO and ROS in macrophages pathway at day 75. However, the inducible form of *NOS* (*NOS2*) is responsible for NO production in macrophages (Fang & Vazquez-Torres 2002) and *NOS2* is subject to different regulatory mechanisms than *NOS1* (Nanetti *et al.* 2004, Pautz *et al.* 2010).

While the upregulation of germ cell-specific genes is a notable pattern in this data set, downregulated genes observed are also likely to play an important role. The fold changes, particularly at day 55, in these genes are greater than those observed in most upregulated genes. Specifically, expression of genes for carrier proteins of

the albumin family *ALB*, *AFP* and *GC* all show large negative fold changes (−48, −12, and −24 respectively, $q < 0.01$ in all cases). These genes are primarily expressed in liver and bind to a number of compounds including steroids and cations such as Ca^{2+} in plasma (Noël *et al.* 2010). The expression pattern and role of the members of the albumin family in the fetal ovary are yet to be fully investigated. It is therefore difficult to translate the differences in gene expression to the changes observed in the fetal ovary. While serum levels of albumin are known to affect levels of intracellular factors such as Ca^{2+} (Fuentes *et al.* 1997), the potential contribution of intracellular albumin in cell homeostasis has also been raised previously (Krishna & Spaniel-Borowski 1989). Downregulation of these genes may serve to increase the availability of the unbound compounds for cellular functions, conceivably an important concept given the important roles of steroids in gonadogenesis (Kezele & Skinner 2003) and Ca^{2+} as discussed previously.

AFP is considered the fetal equivalent of albumin. Expression of *AFP* in the fetus declines with gestational age as expression of *ALB* increases, and by birth, liver expression of *AFP* is negligible (Gabant *et al.* 2002). Despite its abundance early in fetal life, *AFP* is not essential for development (Gabant *et al.* 2002). However, *AFP* is required for female fertility as *AFP*-null mice develop to adulthood, but are infertile (Gabant *et al.* 2002). This infertility is thought to be due to effects on the hypothalamic–pituitary system where the estrogen-binding properties of *AFP* have a neuroprotective effect on the fetal brain (De Mees *et al.* 2006). At day 55 of gestation, the hypothalamic–pituitary system is yet to develop in sheep (Thomas *et al.* 1993), and therefore, it seems unlikely that the differences in *AFP* expression at this age are producing an effect at the hypothalamus or pituitary. However, expression of *AFP* has been observed in ovarian follicles of pregnant rats and the level of expression appears related to the health of the follicle, suggesting some link between this gene and ovarian function (Seralini *et al.* 1986). While potential effects of these differentially expressed albumin family members on ovarian development and subsequent fertility are apparent, further work is required to establish if this differential expression contributes to the phenotypes observed in both fetal ovaries and adult fertility from this cohort of animals.

AFP, *ALB* and *GC* are also members of the HDL/LDL family. Similarly other genes downregulated in this dataset are also classified as members of the HDL/LDL family. *FETUB*, *SERPINA1*, *SERPINA5*, *AMBP*, *AHSG*, *ITIH2*, *APOH*, *AGT*, *HPX* and *VTN* are all downregulated at day 55 with fold changes ranging from −43 to −3, and q values ranging from <0.01 to 0.04. *SERPINA1*, *AMBP*, *AHSG* and *VTN* are also downregulated at day 75 with fold changes ranging from −5.8 to −3.2 and q values ranging from <0.01 to 0.05. Downregulation of the HDL family members (*AGT*, *VTN*, *APOA2*, *APOH*,

SERPINA1, *AHSG*, *AMBP* and *ALB*) is responsible for the predicted effects on the LXR/RXR and FXR/RXR pathways at both day 55 and 75 of gestation. These nuclear receptors serve to regulate multiple metabolic pathways, particularly cholesterol metabolism in response to extracellular signals (Kalaany & Mangelsdorf 2006). Effects on these pathways may indicate changes in cholesterol/steroid metabolism. However, levels of steroid gene expression were similar between groups at both gestational ages and no differences in steroid concentrations in fetal plasma at day 75 were observed in this cohort of animals (ref companion paper). Thus, there is no evidence from this study to suggest that the differences in germ cell development and the phenotypic differences in fertility indicators observed in the female offspring result from differences in steroid production in the developing ovaries.

While differences in HDL/LDL gene expression levels at both gestational ages do not appear to affect steroid production, it is of interest that many of the downregulated HDL/LDL family members also show protease inhibitor activity. These include *FETUB*, *SERPINA1*, *SERPINA5*, *AMBP*, *AHSG*, *SPP2*, and *ITIH* at day 55, and *AMBP*, *AHSG*, *PLG* and *SERPINA1* at day 75. The actions of protease inhibitors can either be destructive (destroying protein function) or positive (activation of a function or pathway) (Law *et al.* 2006). Protease inhibitors control a wide range of biological processes including proliferation (March *et al.* 1993), apoptosis (Mongia *et al.* 2004) and autophagy (Gibellini *et al.* 2012) and inflammation (Safavi & Rostami 2012). Thus, potential for differential expression of protease inhibitors to have impacts on ovarian development is profound. The role of protease inhibitors in fetal ovaries is unclear, and the cell-specific expression pattern for the SDE protease inhibitors has not been published for fetal ovaries. It is also unclear whether the SDE protease inhibitors reported in the current study are able to interact with those proteases involved in germ cell development and thereby influence the establishment of the ovarian reserve, this hypothesis requires further study.

Within the fetal or immature mammalian ovary, the process of autophagy has been implicated in both germ cell death (Escobar *et al.* 2008, Rodrigues *et al.* 2009) and germ cell survival (Gawriluk *et al.* 2011). Tsujimoto proposes that upregulation of the autophagy associated genes *ATG5* and *ATG6*, compared to other *ATG* genes, indicates that autophagy is playing a role in cell death as opposed to cell survival (Tsujimoto & Shimizu 2000). In the current study, the expression of both *ATG5* and *ATG6* is comparable to other *ATG* genes (Supplementary data 1). This suggests that at day 55 and 75 in the sheep fetal ovary autophagy is playing a role in cell survival, a finding in agreement with those of Gawriluk *et al.* in the mouse ovary (Gawriluk *et al.* 2011).

This discussion has largely focused on germ cell development. However, recruitment of granulosa cells during fetal development may play an important

role in establishing the observed effects on fetal germ cell development and subsequently, the increased indicators of fertility in female offspring. It is interesting to note the classical pregranulosa cell marker *LGR5* (Rastetter *et al.* 2014) was not differentially expressed at either day 55 ($q=0.5$) or day 75 ($q=0.9$). Many of the SDE genes are expressed in all cell types, including granulosa cells and pregranulosa cells. Therefore, direct effects of SDE genes on pregranulosa cells are possible. SDE oocyte genes also have the potential to affect the granulosa cells, with *GDF9* (SDE at day 55) being a good example. The role of *Notch-jagged* signaling in germ cell nest breakdown, recruitment of pregranulosa cells and follicle formation has been highlighted recently (Xu & Gridley 2013, Zhao *et al.* 2016). Of further interest, the role of the disintegrin *ADAM10* in *Notch* signaling and pregranulosa cell recruitment has also been reported (Feng *et al.* 2016), with expression of the oocyte-specific genes *JAG1*, *GDF9*, *BMP15* and *NOBOX* all being implicated in this process. In the current study, at day 75, around the time of follicle formation, another disintegrin like gene, *ADAMTS14*, was upregulated (2.5 fold change, $q<0.01$) in the fetal ovaries from dams exposed to restricted nutrition. Of the other genes implicated in pregranulosa cell recruitment via this mechanism, *JAG1* (1.7 fold change, $q<0.01$) and *NOBOX* (2.1 fold change, $q<0.01$) are also upregulated, *GDF9* shows a 1.6-fold upregulation ($q=0.05$) and *BMP15* expression is below the LLD. Thus, in the ovaries of day 75 fetuses from nutritionally restricted dams a pattern of gene expression exists with the potential to enhance pregranulosa cell recruitment and follicle formation.

In conclusion, this study demonstrates changes to the expression pattern of key genes in the fetal ovary following changes to maternal gestational nutrition. In a companion study, female offspring from this cohort of animals showed increased indicators of fertility. Of particular interest, upregulation of genes in fetal ovaries from dams exposed to restricted nutrition associated with NO production and metabolism as well as downregulation of protease inhibitor genes in the same fetal ovaries, offers insights into potential mechanisms whereby altered maternal gestational nutrition may affect fetal ovarian development and subsequently adult fertility.

Conceptually, we propose that the small number of significant fold changes observed in the day 55 fetal ovary transcriptome is a response to allow normal ovarian development in a reduced maternal nutritional environment. While changes to the fetal ovarian transcriptome are apparent at this age, morphological development of the ovary appears normal (ref companion paper). Following cessation of maternal nutritional restriction, we hypothesize gene expression gradually changes to levels consistent with the increased diet, as reflected by the smaller fold changes observed at day

75. It is during this window nutritional of change, (day 55–75) where ovarian gene expression is primed for low maternal nutrition, but the fetal ovary is exposed to high maternal nutrition, that changes to ovarian development are most likely to occur.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0358>.

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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