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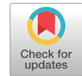
Short communication

# Maternal obesity alters the expression of embryonic regulatory transcripts in the preimplantation ovine conceptus



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ABSTRACT

The influence of exposure to overfeeding-induced maternal obesity around the time of conception on early embryogenesis was examined in the day 14 ovine conceptus. The relative abundance of *FGFR2* and *DNMT1* was influenced by maternal obesity status and conceptus sex, and the abundance of *PPARG* and *PTGS2* transcripts was greater in male conceptuses regardless of the obesity status of the ewe. These observations demonstrated that short-term exposure to maternal obesity impacts early conceptus transcript patterning.

## 1. Introduction

Obesity in humans is associated with several adverse health outcomes, including heart disease, type 2 diabetes and some cancers. Poor diet and lifestyle choices are typically blamed for obesity and its related disorders, however, recent research points to the intrauterine exposure to maternal obesity as a mediating factor predisposing offspring to adverse developmental outcomes. The programming of offspring exposed to intrauterine stressors is referred to the Developmental Origins of Adult Health and Disease (DOHAD). This developmental phenomenon highlights the interaction between the maternal and embryonic systems, and the consequential health and development of offspring. Initial studies examining DOHAD focused on the effects of under nutrition on subsequent offspring development, but the adverse effects of fetal exposure to maternal over nutrition on offspring outcomes in humans and other mammalian species has also been observed. Adverse health outcomes observed include increased birth and postnatal body weights, insulin resistance, hyperlipidemia, hypertension, reduced bone mineral content and fatty liver disease.

The period surrounding conception (peri-conception) appears to be especially sensitive to the adverse effects of maternal obesity. Various critical embryonic events during the peri-conception period including oocyte maturation, fertilization, rapid demethylation and remethylation of the embryonic genome, embryonic genome activation, and formation of multipotent embryonic and extraembryonic lineages [1]. Work by McMillen and colleagues utilized an embryo transfer model in sheep to examine how periconception exposure to maternal obesity affects postnatal lamb development (reviewed in [2]). For this work, obese ewes generated by overfeeding and normal-fed, lean counterparts were superovulated and bred, and embryos were harvested at day 6–7 after breeding and transferred into non-obese ewes. Peri-conceptional exposure to obesity did not affect lamb birth weights or weights at 4 months of age, but this short-term exposure to obesity before embryo transfer increased visceral fat mass in growing lambs [3]. This effect was dependent on lamb sex, with peri-conceptional obesity exposure affecting fat deposition in ewe lambs (females) but not ram lambs (male) [3]. The aim of this study was to determine whether peri-conceptional exposure to maternal obesity induced by overfeeding adversely impacts the abundance of transcripts involved with early development of embryonic and extraembryonic membranes (collectively referred to as the 'conceptus'). Transcripts of interest included *ASCL2, CDX2, HAND1, APOA1, GATA4, FGFR2, PPARG, PTGS2, IFNT, DNMT1, DNMT3a, and DNMT3b*. The working hypothesis is that maternal obesity can alter the expression of transcripts that regulate early conceptus development and function in peri-implantation ovine conceptuses.

*Abbreviations:* APOA1, apolipoprotein A1; ASCL2, Achaete-Scute Family BHLH Transcription Factor 2; CDX2, caudal type homeobox 2; CIDR, controlled intravaginal drug release device; CL, corpora lutea; DNMT(13a/b), DNA methylatransferase; DOHAD, developmental origins of adult health and disease; FGF, fibroblast growth factor; FGFR2, FGF receptor 2; GATA4, 'GATA' sequence transcript factor 4; HAND1, heart and neural crest derivative 1; IFNT, interferon-tau; PPARG, peroxisome proliferator-activated receptor gamma; PRE, primitive endoderm; PTGS2, prostaglandin-endosomal synthase 2; qRT-PCR, quantitative reverse transcription polymerase chain reaction; TE, trophectoderm; RPS9, ribosomal protein S9; SEM, standard error of the mean

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## 2. Materials and methods

### 2.1. Animal use

All animal work was completed in accordance and with the approval of the Virginia Tech Institutional Animal Care and Use Committee. Dorset ewes (2–5 years of age) were assigned randomly to lean or obese groups. The average starting body weight for ewes was 74.9 ± 2.3 Kg (n = 27 ewes). The obese state was induced by feeding 1 kg shelled corn/day and providing ad libitum exposure to high quality pasture in the summer and orchard grass hay in the fall and winter months. Ewes that achieved a body condition score ≥4 (scale of 1–5) were chosen for further study. Body condition scoring is a hallmark indicator of subcutaneous and visceral adipose size in sheep, and a score ≥4.0 indicate excessive fatness that is considered obese [4]. Normal fed, lean ewes were kept on a maintenance diet composed of previously grazed pasture in the summer months and poor-quality hay in the fall and winter months. This yielded a body condition score of 2.5–3.

Once a body condition score ≥4 was obtained in the overfed ewes (~4 months), animals underwent an estrous synchronization protocol that began with insertion of progesterone-containing controlled intravaginal drug release device (CIDR) (Pfizer, New York, NY) and intramuscular injection of a gonadotrophin releasing hormone agonist (Cystorelin™; Merial, Lyon, France; 50 μg). Seven days later, the CIDR was removed and a prostaglandin F2α agonist was injected intramuscularly (Lutalyse™; Zoetis, Parsippany, NJ; 15 mg; IM). Ewes were then bred to genetically-related Dorset rams (three-quarter siblings).

### 2.2. Conceptus collections

Ewes were sacrificed on day 14 of gestation (day 0 = day of breeding). Harvesting conceptuses at day 14 of development permitted us to capture a unique stage in conceptus development. Unlike many other mammals, ruminant conceptuses do not implant after blastocyst formation and zona pellucida hatching. Rather, they remain free-floating in the uterus for an additional 5–8 days before making firm connections with the uterine lining. At day 14 of development, ovine conceptuses have undergone an elongation process that provides ample amounts of extraembryonic membranes, and specifically TE and PRE, but they have not yet made firm connections with the uterine lining. This permitted us to easily flush the conceptuses out of the uterus without contamination with uterine tissue.

Body weight was recorded at the time of sacrifice. The uterus was excised by mid-ventral dissection. Each uterine horn was flushed with 30 mL Dulbecco's phosphate-buffered saline [pH 7.2] (Gibco, Gaithersburg, MD) to recover conceptuses. Individual conceptuses were teased apart and each length was recorded. Also, the number of corpora lutea (CL) was recorded and used to determine the percentage pregnant per ovulation. Individual conceptuses were snap-frozen in liquid nitrogen and stored at -80°C.

### 2.3. Conceptus sex determination

Conceptus DNA and RNA were isolated using the AllPrep Mini Kit (Qiagen, Hilden, Germany). DNA quantity was examined using a Nanodrop Spectrophotometer ND-1000 (Nanodrop; Thermo Fisher). Conceptus sex was determined using a previously described PCR-based approach [5] using GoTaq Green Master Mix (Promega, Madison, WI). Samples underwent an initial 5 min denaturation step at 95°C, followed by 40 cycles of 95C, 56°C and 72°C, and ending with a 5-min polishing step at 72°C. Samples were then digested with Sac1 and electrophoresed on a 1% [w/v] agarose gel. DNA was detected using SYBR Safe DNA gel stain (ThermoFisher, Waltham, MA). Male conceptuses were identified by the presence of 3 bands while females contained 2 bands.

**Table 1**
Forward and reverse primers used in qRT-PCR analysis.

| Gene | Primer sequence (5′ to 3′)[a] | Reference |
|---|---|---|
| *IFNT* | F: ATGGCCTTCGTGCTCTCTCT<br>R: CCTGGCATCCAGCATGAGTC | – |
| *CDX2* | F: GCCACCATGTACGTGAGCTAC<br>R: ACATGGTATCCGCCGTAGTC | – |
| *HAND1* | F: CAAGGACGCACAGGCTGGCGA<br>R: CACTGGTTTAGCTCCAGCGC | – |
| *ASCL2* | F: GCTGCTCGACTTCTCCAG<br>R: CGGAACGAGGAACACGG | – |
| *GATA4* | F: GGTTCCCAGGCCTCTTGCAATGCGG<br>R: AGTGGCATTGCTGGAGTTACCGCTG | [11] |
| *PTGS2* | F: TCCGCCAACTTATAATGTGCAC<br>R: GGCAGTCATCAGGCACAGGA | [12] |
| *APOA1* | F: CTCTGAGTTCCACATCGCCA<br>R: TGGCCAGCAGTCTAATCAGC | – |
| *DNMT1* | F: AAGTCAAACCAAAGAACC<br>R: TTCTCATCAGAGACTTGTGG | [13] |
| *DNMT3a* | F: TGTACGAGGTACGGCAGAAGTG<br>R: GGCTCCCACAAGAGATGCA | [14] |
| *DNMT3b* | F: GACGTAGAGGGCAGAGATGC<br>R: ATCACCAAACCACTGGACCC | – |
| *FGFR2* | F: CCTGCGGAGACAGGTAACAG<br>R: GCAGCTCATACTCGGAGACC | – |
| *PPARG* | F: TAGGTGTGATCTTAACTGT<br>R: CTGATGGCATTATGAGAC | [15] |
| *RPS9* | F: CAAGTCCATCCACCATGCCC<br>R: GACGGGATGTTCACCACCTG | – |

The dash for references indicates that primers were made and validated in-house using NCBI software.
[a] Forward (F) and reverse (R) primer sequences.

### 2.4. Quantitative RT-PCR

Total cellular RNA extracted using the previously described AllPrep system were incubated with RNAse-free DNAse for 30 min at 37°C (Life Technologies, Carlsbad, CA). RNA quantity was examined using a Nanodrop Spectrophotometer ND-1000 (Nanodrop; Thermo Fisher). All samples contained adequate quality for quantitative (q) RT-PCR analysis (< 1.8 $A_{260/280}$ ratio). Samples were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). PCR was performed using the SybrGreen detection system (Life Technologies) in combination with primers for selected transcripts (Table 1). Primers were designed either based on published reports or by using NCBI-BLAST Primer Design Software and synthesized by Life Technologies. *RPS9* was used as the internal reference control. Its expression was not affected by obesity or conceptus sex (data not shown). PCR was completed with an Eppendorf Realplex4 Mastercycler (Hamburg, Germany). Samples we initially denatured at 95°C for 10 min and then underwent 40 cycles of denaturation (95°C, 15 s), annealing (57°C, 15 s), and synthesis (68°C, 20 s). A melting curve analysis was completed to verify amplification of a single product. Each PCR sample was run in triplicate, and a fourth sample lacking the reverse transcriptase was included as a negative control. Gene expression data is represented by fold-change relative to the treatment that contained the lowest expression value.

### 2.5. Statistical analyses

All data were analyzed by least squares analysis of variance using the general linear model of the Statistical Analysis System (SAS Institute, Inc., Cary, NC, USA). The relative RNA abundance data was log-transformed before analysis. Differences between individual treatment means were partitioned further by pair-wise comparisons (PDIFF [probability of difference] analysis in SAS). Results were presented as the arithmetic mean ± standard error of the mean (SEM).

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