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Research Article

Influence of estradiol on bovine trophectoderm and uterine gene transcripts around maternal recognition of pregnancy†

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Abstract

Embryo survival and pregnancy success is increased among animals that exhibit estrus prior to fixed time-artificial insemination, but there are no differences in conceptus survival to d16. The objective of this study was to determine effects of preovulatory estradiol on uterine transcriptomes, select trophectoderm (TE) transcripts, and uterine luminal fluid proteins. Beef cows/heifers were synchronized, artificially inseminated (d0), and grouped into either high (highE2) or low (lowE2) preovulatory estradiol. Uteri were flushed (d16); conceptuses and endometrial biopsies ($n = 29$) were collected. RNA sequencing was performed on endometrium. Real-time polymerase chain reaction (RT-PCR) was performed on TE ($n = 21$) RNA to measure relative abundance of *IFNT*, *PTGS2*, *TM4SF1*, *C3*, *FGFR2*, and *GAPDH*. Uterine fluid was analyzed using 2D Liquid Chromatography with tandem mass spectrometry-based Isobaric tags for relative and absolute quantitation (iTRAQ) method. RT-PCR data were analyzed using the MIXED procedure in SAS. There were no differences in messenger RNA (mRNA) abundances in TE, but there were 432 differentially expressed genes (253 downregulated, 179 upregulated) in highE2/conceptus versus lowE2/conceptus groups. There were also 48 differentially expressed proteins (19 upregulated, 29 downregulated); 6 of these were differentially expressed (FDR < 0.10) at the mRNA level. Similar pathways for mRNA and proteins included: calcium signaling, protein kinase A signaling, and corticotropin-releasing hormone signaling. These differences in uterine function may be preparing the conceptus for improved likelihood of survival after d16 among highE2 animals.

Summary sentence

Preovulatory estradiol did not impact conceptus survival to d16; however, it did influence uterine gene/protein expressions related to adhesion, endometrial remodeling, metabolism, and immune regulation, which may explain improved pregnancy success.

Key words: maternal recognition of pregnancy, preovulatory estradiol, proteomics, transcriptomics.

Introduction

An adequate uterine environment is necessary for maternal and conceptus communication. It must provide sufficient nutrients and endocrine conditions for the establishment and maintenance of pregnancy. In cattle, elongating conceptuses are free floating in the uterus until time of attachment (d20). During this time of elongation, conceptuses are relying on the maternal environment and secretions from the uterus for growth, development, and survival. These secretions from uterine epithelium are termed uterine histotroph. It is composed of a complex mixture of enzymes, growth factors, cytokines, lymphokines, hormones, amino acids, proteins, and glucose [1]. These nutrients stimulate the nutrient-sensing signaling pathway to increase translation of messenger RNA (mRNA), which is critical for conceptus development [2]. Cell signaling through this pathway also stimulates cell migration, invasion, and cell growth and proliferation [3].

When Gray et al. [4] placed uterine gland knockout sheep with fertile rams, no pregnancies were identified on d25 after insemination. Additionally, blastocyst growth into an elongated bovine conceptus has not been able to be duplicated in vitro [5]. These studies demonstrate that endometrial glands and their secretions are necessary for pregnancy establishment and conceptus development.

Advancements in transcriptomic and proteomic technologies have allowed researchers to gain a better understanding of the uterine milieu and biological mechanisms associated with early pregnancy in ruminants. Interferon tau, progesterone, estradiol, prostaglandins, and cortisol have been reported to act as key regulators in the uterus [6]. Specifically, they can influence gene expression and protein abundances within uterine luminal fluid (ULF) that aid in elongation, recognition of pregnancy, implantation, and placentation.

In particular, preovulatory estradiol impacts follicular growth, oocyte maturation, sperm transport, uterine environment, and embryo survival/development [7]. Cows in standing estrus prior to fixed-time artificial insemination (AI) have increased embryo survival and greater pregnancy success than nonestrus animals [8]. Specifically, when ovariectomized beef cows were treated with estradiol (cypionate or benzoate), embryo survival was increased to d29 of pregnancy compared with cows that had no exposure to estradiol [9]. Furthermore, cows that received exogenous estradiol only lost 35% of their existing pregnancies, whereas control animals lost 75% of their existing pregnancies [9]. Our laboratory has determined that there were no differences in conceptus survival to d16 of pregnancy between highE2 and lowE2 animals based on conceptus recovery rates, apoptosis of trophectoderm (TE) cells, and interferon tau concentrations in ULF [10]. However, there were differences in select glucose transporter mRNA abundances in caruncular and intercaruncular endometrium [10]. Despite there being extensive research regarding the importance of preovulatory estradiol during early pregnancy, little is known about its impact on uterine and TE transcriptomes and proteomes around maternal recognition of pregnancy. The objectives of the current study were to: 1) examine effects of preovulatory estradiol on critical genes and pathways associated with early pregnancy in cattle and 2) examine the impact of preovulatory estradiol on ULF protein abundances. We hypothesize that differences in uterine environment will have a greater impact on pregnancy success than differences in conceptus developmental competence on d16 of pregnancy in beef cattle.

Materials and methods

Animals

All procedures were approved by the South Dakota State University Institutional Animal Care and Use Committee and US Meat Animal Research Center (USMARC) Animal Care and Use Committees in accordance with the Federation of Animal Science Societies (FASS) guidelines for the care and use of agricultural animals in research.

Treatments

Angus-crossed beef cows/heifers at the South Dakota State University Beef Breeding Unit (Rep 1: $n = 30$, Rep 2: $n = 40$) and the USMARC (Rep 3: $n = 20$) were synchronized with a CO-Synch protocol: Gonadotropin releasing hormone (GnRH) administered (100 µg as 2 mL of Factrel i.m.; Pfizer Animal Health, Madison, NJ) on d9, followed by PGF2 alpha (PG; 25 mg as 5 mL of Lutalyse i.m.; Pfizer Animal Health, Madison, NJ) on d −2, and on d −0, cows were administered GnRH (100 µg as 2 mL of Factrel i.m.; Pfizer Animal Health, Madison, NJ) and artificially inseminated. Estrus was monitored visually from d0 through d3 with the aid of EstroTect patches (Western Point, Inc., Apple Valley, MN). Only animals that ovulated following the GnRH injection at fixed-time AI were utilized. Animals were grouped into either highE2 or LowE2 based on preovulatory estradiol concentrations (replicates 1 and 2) and expression of estrus (all replicates). The threshold estradiol concentration that distinguished the two groups was 4.9 pg/mL. Previous research used a similar cutoff when evaluating changes in ovarian function associated with concentrations of estradiol before a GnRH-induced ovulation in beef cows [10, 11].

Ultrasonography and detection of estrus

For replicates 1 and 2, follicular dynamics were assessed by transrectal ultrasonography using an Aloka 500 V ultrasound with a 7.5 MHz linear probe (Aloka, Wallingford, CT) on d −9, 0, and 3 to characterize follicular development and ovulation. All follicles on each ovary >8 mm in diameter were recorded. Ovulation was defined as the disappearance of a previously recorded large follicle and confirmed by changes in circulating concentrations of progesterone.

Blood sampling and radioimmunoassays

For replicates 1 and 2, blood samples were collected by venipuncture of the jugular vein into 10 mL Vacutainer tubes (Fisher Scientific, Pittsburgh, PA). For the first replicate, blood was collected on d −2, −1, 0, then every other day through d16. For the second replicate, blood was collected on d −2, −1, 0, then every other day through d15. Blood was centrifuged at 1200 × g for 30 min at 4°C, and plasma was collected and stored at −20°C. Radioimmunoassays were performed on plasma samples to determine circulating progesterone concentrations [12]. Intra- and interassay Coefficient of Variation (CV) were 4.9% and 7.5% and 6.0% and 13.2% for replicates 1 and 2, respectively, and assay sensitivity was 0.4 ng/mL. Plasma concentrations of estradiol were determined within replicate by a single assay [13]. Intra-assay CVs were 5.03% and 4.76%, for replicates 1 and 2, respectively. Assay sensitivity was 0.5 pg/mL.

Conceptus recovery

In replicate 1, uteri were flushed nonsurgically using a modified Foley catheter on d16. The catheter was inserted into the vagina through the cervix, and into the uterus. Animals were flushed with

Table 1. Genes, primer sequences, annealing temperatures, and product sizes for endometrium genes amplified during RT-PCR.

Gene	Primer	Primer sequence	T_a (°C)	Product Size (bp)	References
DDX58	Forward	5'-GGAAGACCCTGGACCCTACCT-3′	60	72	Song et al., 2011 [52]
	Reverse	5'-TATACTGCACCTCTTCCTCCCTAAA-3′			
ISG15	Forward	5'-GGTATCCGAGCTGAAGCAGTT-3′	60	293	Green et al., 2010 [53]
	Reverse	5'-ACCTCCCTGCTGTCAAGGT-3′			
OXTR	Forward	5'-ACGGTGTCTTCGACTGCTG-3′	60	110	Bauersachs et al., 2006 [18]
	Reverse	5'-GGTGGCAAGGACGATGAC-3′			
PARP12	Forward	5'-CAACGTGAGCGTGCTGAAAA-3′	60	90	Primer-Blast
	Reverse	5'-AAGAGCAAGGGGTCGTTCTG-3'			
RSAD2	Forward	5'-GTGGTTCCAGAAGTACGGTGA-3′	60	103	Boruszewska et al., 2017 [54]
	Reverse	5'-CTTCTTTCCTTGACCACGGC-3'			
XAF1	Forward	5'-GAGGAGGCTCTGAGCTTGC-3′	64	143	Groebner et al., 2010 [55]
	Reverse	5'-GCAGAGAAAGATGTCCGTCC-3'			
PRSS8	Forward	5'-ATGGGATAGGAGCCGTTGTG-3′	60	161	Primer-Blast
	Reverse	5′- CTGATCGGACACGAGAGAGC-3'			
CXCL10	Forward	5'-CACTCCTCAACTCTTCAGGC-3′	50	262	Imakawa et al., 2006 [56]
	Reverse	5'-CCATTCCTTTTCATTGTGGC-3'			
IDO1	Forward	5'-GGGCCCATGACTTATGAGAA-3′	60	107	Groebner et al., 2011 [57]
	Reverse	5'-GAGGCAGCTGCTATTTCCAC-3'			
MUC13	Forward	5'-ACGGGCTGGTGAGACCAAAACC-3′	60	116	Forde et al., 2013 [35]
	Reverse	5'-GCAGTCAGCTGTCCCGTTGC-3'			
CLDN4	Forward	5'-CTTCTTCCATCCTTCCTGCTC-3′	64	164	Riedmaier et al., 2014 [58]
	Reverse	5'-TCTAAACCTGTCCGTCCACTC-3'			
FABP3	Forward	5'-GAGATCAGCTTCAAGCTGGGA-3′	60	121	Mansouri-Attia et al., 2009 [19]
	Reverse	5'-CTTGTCCATTCCACTTCTGCAC-3'			
GAPDH	Forward	5'-GATTGTCAGCAATGCCTCCT-3′	60	94	Han et al., 2006 [59]
	Reverse	5'-GGTCATAAGTCCCTCCACGA-3'			

Table 2. Genes, primer sequences, annealing temperatures, and product sizes for TE genes amplified during RT-PCR.

Gene	Primer	Primer Sequence	T_a (°C)	Product size (bp)	References
IFNT	Forward	5'- GCTATCTCTGTGCTCCATGAGATG-3′	58	359	Shorten et al., 2018 [60]
	Reverse	5'-AGTGAGTTCAGATCTCCACCCATC-3'			
PTGS2	Forward	5'-GCATTCTTTGCCCAGCACTTCACCC-3′	58	418	Lussier et al., 2017 [61]
	Reverse	5'-CTATCAGGATTAGCCTGCTTGTCTGG-3'			
TM4SF1	Forward	5'- TCTTCTCCGGTATCCTGGGA-3′	56	155	Primer3
	Reverse	5′- TCCAATGAGTGCAGCCAGTA-3'			
C3	Forward	5'-AGAACATCTGGGTCAAGGGG-3′	56	201	Primer3
	Reverse	5'-ATCATGTTCTGCTCCCCACA-3'			
FGFR2	Forward	5'-CACCACGGACAAAGAAATTG-3′	58	113	Akbarinejad et al., 2016 [62]
	Reverse	5'-ATGCAGAGTGAAAGGATATCCC-3'			
GAPDH	Forward	5'-GATTGTCAGCAATGCCTCCT-3′	60	94	Han et al., 2006 [59]
	Reverse	5'-GGTCATAAGTCCCTCCACGA-3'			

100 mL of flush media to maintain a constant volume. Uteri were massaged, and fluid drained through a filter above a conical tube. Flush media was assessed under a microscope at 10× to determine whether a conceptus was present or not. If no conceptus was recovered, additional flush media was added, and this additional media was collected separately. TE (highE2: $n = 6$, lowE2: $n = 3$) was separated from embryo proper, snap froze and was then stored at −80°C.

In replicate 2, reproductive tracts were collected from the abattoir immediately following slaughter on d16 and kept on ice. An incision was made at the anterior end of the uterine horn contralateral to the corpus luteum; a plastic tube was placed in the uterine tip and sutured to prevent any fluid loss while the other horn was clamped off. Uterine horns were flushed with 30 mL of flush media and then massaged for equal fluid distribution in the uterus. Uterine flush fluid was then collected in a 50-mL conical tube and examined under a microscope at 10× to determine if a conceptus was present. TE (highE2: $n = 6$, lowE2: $n = 3$) was separated from embryo proper, was snap frozen, and was then stored at −80°C.

In replicate 3, reproductive tracts were collected from the abattoir at USMARC immediately following slaughter on d16. An incision was made at the anterior end of the uterine horn contralateral to the corpus luteum; a plastic tube was placed in the uterine tip and sutured to prevent any fluid loss while the other horn was clamped off. Uterine horns were flushed with 20 mL of flush media and then

massaged for equal fluid distribution in the uterus. Uterine flush fluid was then collected in a 50-mL conical tube and examined under a microscope at 10× to determine if a conceptus was present. TE (highE2: $n = 8$, lowE2: $n = 2$) was separated from embryo proper and was then stored at −80°C.

Endometrium collection, RNA extraction, and RNA sequencing

In replicate 1, endometrium from midway down the ipsilateral uterine horn was collected via a Jackson Uterine Biopsy instrument (Universal Surgical Instruments and Better Surgical Instrumentation; $n = 23$). We were unable to pass the biopsy tool on some heifers in the study. In replicate 2 ($n = 28$) and replicate 3 ($n = 20$), the ipsilateral uterine horn was cut anterior to the bifurcation, and endometrial tissue was collected midway down the ipsilateral horn.

For all replicates, total cellular RNA was extracted using the Qiagen RNeasy Plus Mini Kit (Austin, TX) following the manufacturer's instructions. Pure RNA was dissolved in nuclease free water, and a spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to determine RNA concentration for each sample. RNA integrity was then determined using an Agilent RNA Screen Tape System. Only RNA samples (lowE2/noconceptus: $n = 8$, lowE2/conceptus: $n = 3$, highE2/noconceptus: $n = 7$, highE2/conceptus: $n = 11$) with a RNA integrity number (RIN) > 7 were sent to the University of Minnesota Genomic Center for total RNA sequencing. They created 29 dual-indexed TruSeq-stranded mRNA libraries. All libraries were combined into a single pool and were sequenced across two lanes of a NovaSeq S2, 2 × 50-bp run. There were approximately 24 M reads generated for each sample. Illumina Basis QC analysis was performed on all paired-end sequences. All libraries had mean quality scores that were ≥30, and the pools were gel sized selected to have inserts that were approximately 200 base pairs long.

Endometrium RT-PCR validation

Twelve differentially expressed genes (DEGs) according to sequencing were selected for validation using real-time polymerase chain reaction (RT-PCR); additional animals were added to the validation population. Total cellular RNA ($n = 55$; lowE2/noconceptus: $n = 17$ lowE2/conceptus: $n = 6$, highE2/noconceptus: $n = 15$, highE2/conceptus: $n = 17$) was diluted to 70 ng/μL (280 ng/reaction), and RT-PCR was performed in duplicate using iScript One-Step RT-PCR Kit with SYBR Green (BioRad) and Stratagene MX 3000P QPCR machine. Expression *of DDX58, ISG15, OXTR, PARP12, RSAD2, XAF1, PRSS8, CXCL10, IDO1, MUC13, CLDN4,* and *FABP3* was measured using the primers in Table 1, and *GAPDH* was used as a reference gene. All primers were diluted to a concentration of 10 μM. Each plate contained negative controls to assure no background contamination. The PCR program was 10 min at 50°C and 1 min at 95°C for inactivation of reverse transcriptase. Transcription was then followed by 15 s at 95°C for melting and 30 s at the designated annealing temperature (Table 1) for 40 cycles. All CVs were less than 20%. Amplicons were electrophoresed on 2% agarose gels to determine product size and were verified for identity by sequencing (Iowa State Genomics Core).

TE RNA extraction and RT-PCR

Total cellular RNA was extracted using the Qiagen RNeasy Mini Kit (Austin, TX), following the manufacturer's instructions with some modifications. TE tissue (Rep 1: $n = 7$, Rep 2: $n = 5$, Rep 3: $n = 9$) was lysed in RLT buffer using a 22-gauge needle and vortexed. After the first RW1 wash solution step, 80 μL of DNase solution was added directly to the membrane and incubated for 15 min at room temperature. Pure RNA was dissolved in nuclease-free water, and a spectrophotometer was used to determine RNA concentration for each sample. The RNA samples were stored at −80°C. The RNA (190 ng) was reverse transcribed into cDNA via the BioRad iScript cDNA synthesis kit following the manufacturer's instructions. RT-PCR was then performed on TE cDNA (6 ng) in duplicate using BioRad iTaq Universal SYBER Green Supermix and BioRad C1000 Touch CFX96 Real Time System.

Expression of *IFNT, PTGS2, TM4SF1, C3,* and *FGFR2* was measured using the primers in Table 2 and *GAPDH* used as a reference gene. All primers were diluted to a concentration of 10 μM. Each plate had negative controls to assure no background contamination. The PCR program was 5 min at 95°C for melting, 15 s at the given annealing temperature (Table 2) and 15 s at 70°C for extension, for 40 cycles. All CVs were less than 20%. Amplicons were electrophoresed and were verified for identity by sequencing (Iowa State Genomics Core).

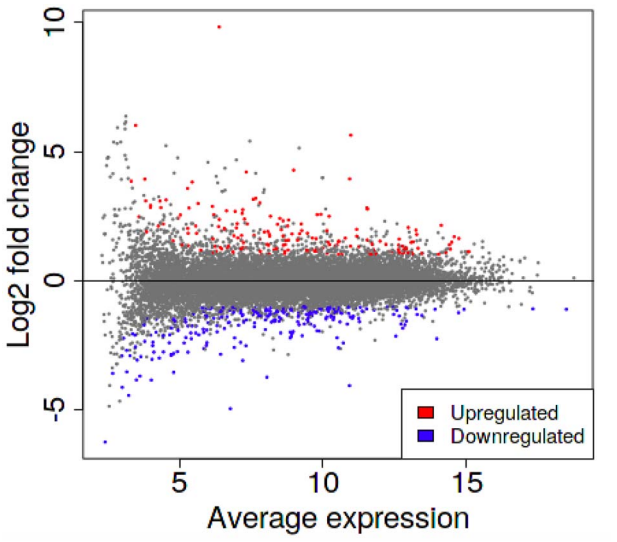

Figure 1. MA plot depicting DEGs among the highE2/conceptus versus lowE2/conceptus comparison.

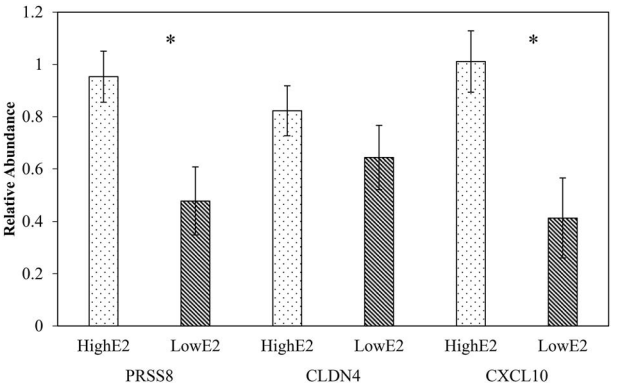

Figure 2. Endometrium mRNA abundances for *PRSS8, CLDN4,* and *CXCL10* on day 16 among highE2 and lowE2 animals. HighE2 animals had increased *PRSS8* ($P = 0.005$) and *CXCL10* ($P = 0.003$) transcript abundances compared with lowE2 animals; *CLDN4* expression was not different (* $P < 0.05$).

Table 3. DEGs in the endometrium of highE2 versus lowE2 animals with a conceptus.

Biological function	Gene abbreviation	Gene name	Log2Fold change	FDR
Endometrial remodeling	*MYLK*	Myosin light chain kinase	-1.17	2.03E-07
	ADAM12	ADAM metallopeptidase domain 12	-1.67	0.00377
	TAGLN	Transgelin	-2.13	0.00207
	ACTA2	Actin, alpha 2, smooth muscle	-1.83	0.00604
	MYL9	Myosin light chain 9	-1.02	0.00597
	COL1A2	Collagen type I alpha 2 chain	-1.08	0.00666
	COL3A1	Collagen type III alpha 1 chain	-1.10	0.00976
	PRSS8	Protease, serine 8	2.06	0.00756
	GRN	Granulin precursor	1.13	0.000946
	HSPE	Heparanase	-2.62	0.02
	ADAMTS15	ADAM metallopeptidase, thrombospondin type 1 motif 15	-1.39	0.0236
	LGMN	Legumain	1.40	0.0291
Metabolic	*FABP3*	Fatty acid binding protein 3	2.82	0.00604
	SLC2A1	Solute carrier family 2 member 1	1.64	0.0357
	SLC5A5	Solute carrier family 5 member 5	3.13	0.00606
	SLC27A5	Solute carrier family 27 member 5	1.40	0.00013
	LPL	Lipoprotein lipase	-2.57	0.00509
	SLC38A4	Solute carrier family 38 member 4	-1.18	0.0217
	FAAH	Fatty acid amide hydrolase	-2.93	0.0233
	AMPD3	Adenosine monophosphate deaminase 3	1.80	0.03
	FBP1	Fructose-bisphosphatase 1	3.19	0.0312
	ACO2	Aconitase 2	1.23	0.0327
	SLC27A2	Solute carrier family 27 member 2	1.82	0.049
	SLC7A2	Solute carrier family 7 member 2	-1.06	0.049
Adhesion	*CLDN4*	Claudin 4	1.71	0.0494
	F5	Coagulation factor V	1.34	0.0147
	MUC13	Mucin 13, cell surface associated	1.53	0.0276
	ITGA3	Integrin subunit alpha 3	1.21	0.0173
	CDH4	Cadherin 4	-1.74	0.0186
	CLEC4F	C-type lectin domain family 4 member F	2.36	0.0208
	TROAP	Trophinin associated protein	-1.44	0.0217
	ITGB5	Integrin subunit beta 5	1.49	0.0265
Immune regulation	*IDO1*	Indoleamine 2,3-dioxygenase 1	2.50	0.0182
	BPI	Bactericidal/permeability-increasing protein	1.48	0.0386
	BOLA-NC1	Nonclassical MHC class I antigen	1.03	0.00234
	C2	Complement C2	1.51	0.0488
	CFB	Complement factor B	2.20	0.00001
	CR2	Complement C3d receptor 2	-2.39	0.00795
	C1QL2	Complement C1q like 2	4.21	0.00005
	OAS2	2'-5'-oligoadenylate synthetase 2	1.58	0.0437
	S100A12	S100 calcium binding protein A12	1.66	0.0213
	CD48	CD48 molecule	1.27	0.00055
	MIC1	Major histocompatibility class I related protein	1.82	0.00352
	CXCL10	C-X-C motif chemokine ligand 10	2.78	0.00133
	CXCL11	C-X-C motif chemokine ligand 11	1.93	0.0208

Mass spectrometry

There were 28 samples of ULF that were used to make a total of eight pools that were sent to the Mass Spectrometry Facility at the University of Minnesota. There were two independent pools made for each of the following groups: lowE2/noconceptus, lowE2/conceptus, highE2/noconceptus, and highE2/conceptus. Each animal was represented equally within the appropriate pool. Protein quantification was conducted using a 2D Liquid Chromatography with tandem mass spectrometry (LC MS/MS)-based 8plex Isobaric tags for relative and absolute quantitation (iTRAQ) quantitative method. Samples were pooled, reduced, alkylated by methyl methanethiosulfonate digested with trypsin, and labeled with iTRAQ reagents. Scaffold Q+ (version Scaffold_4.8.4, Proteome Software Inc., Portland, OR) was used to quantitate label-based quantitation (iTRAQ) peptide and protein identifications.

Data analyses

For RNA sequencing, quality reads were mapped to the bovine reference genome ARS-UCD1.2 using kallisto [14]. Genes were filtered at a cutoff of 0.5 counts per million before fitting data into a negative binomial distribution. Differential expression analysis was conducted using the bioconductor package, DESeq2. Genes

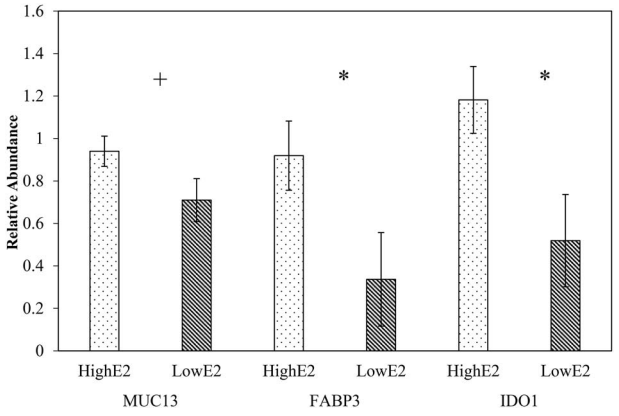

Figure 3. Endometrium mRNA abundances for *MUC13, FABP3*, and *IDO1* on day 16 among highE2 and lowE2 animals. HighE2 animals had increased *FABP3* ($P = 0.04$) and *IDO1* ($P = 0.02$) transcript abundances compared with lowE2 animals. There was a tendency for highE2 animals to have increased *MUC13* ($P = 0.07$) transcript abundance in the endometrium on day 16 compared with lowE2 animals ($^{*}P > 0.05$, $^{+}P < 0.10$).

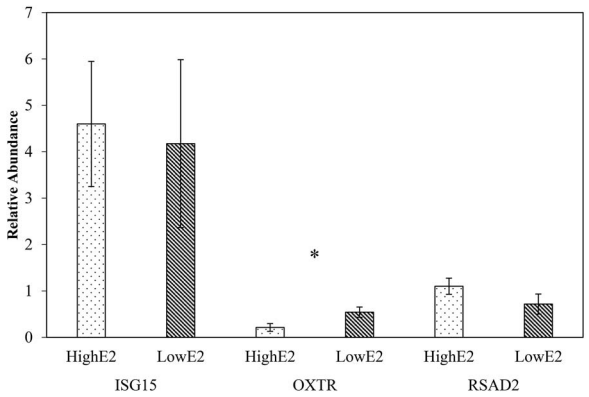

Figure 4. Endometrium mRNA abundances for *ISG15, OXTR*, and *RSAD2* on day 16 among highE2 and lowE2 animals. HighE2 animals had decreased OXTR ($P = 0.02$) transcript abundance compared with lowE2 animals. There was no difference in *ISG15* and *RSAD2* transcript abundances in the endometrium on day 16 ($^{*}P < 0.05$).

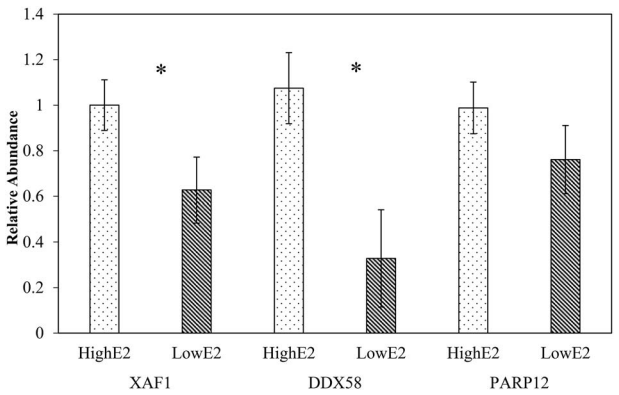

Figure 5. Endometrium mRNA abundances for *XAF1, DDX58*, and *PARP12* on day 16 among highE2 and lowE2 animals. HighE2 animals had increased *XAF1* ($P = 0.02$) and *DDX58* ($P = 0.0007$) transcript abundances compared with lowE2 animals. There was no difference in *PARP12* transcript abundances in the endometrium on day 16 ($^{*}P > 0.05$).

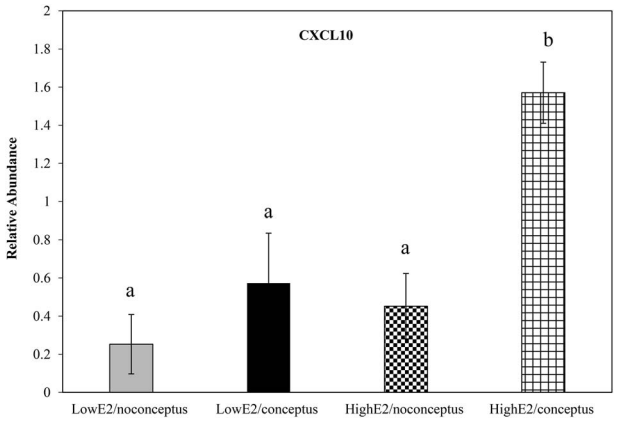

Figure 6. Preovulatory estradiol exposure and conceptus presence interaction ($^{ab}P = 0.04$) on *CXCL10* mRNA abundance in endometrium on day 16 of pregnancy.

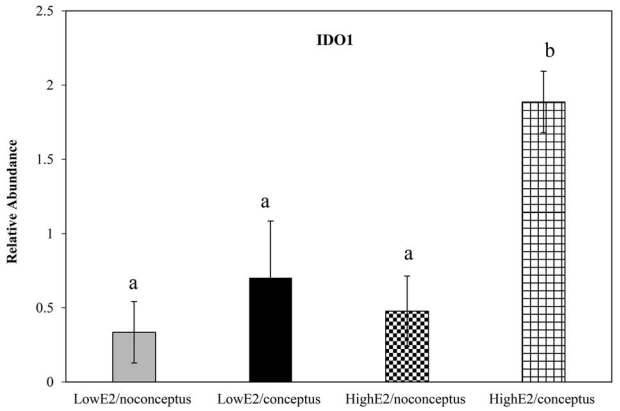

Figure 7. Preovulatory estradiol exposure and conceptus presence interaction ($^{ab}P = 0.06$) on *IDO1* mRNA abundance in endometrium on day 16 of pregnancy.

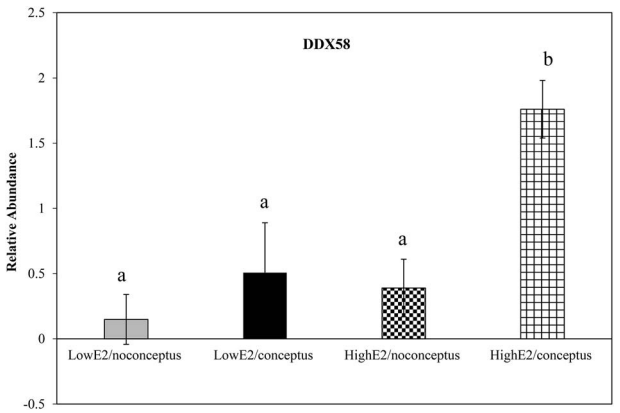

Figure 8. Preovulatory estradiol exposure and conceptus presence interaction ($^{ab}P = 0.06$) on *DDX58* mRNA abundance in endometrium on day 16 of pregnancy.

were considered differentially expressed if false discovery rate (FDR) < 0.05 and fold change was > 2. TE data were analyzed using the MIXED procedure in SAS with *GAPDH* being used as a reference gene. Endometrium RT-PCR data were analyzed using the MIXED procedure in SAS with conceptus presence, preovulatory estradiol

Table 4. DEPs downregulated in highE2/conceptus animals compared with lowE2/conceptus animals.

Abbreviation	Protein name	Log2Fold change	FDR
UTMP	Uterine milk protein	-2.02	0.00027
ORM1	Alpha-1-acid glycoprotein	-1.31	<0.0001
ANXA8	Annexin A8	-1.23	<0.0001
ANXA1	Annexin A1	-0.89	<0.0001
FGG	Fibrinogen gamma-B chain	-0.88	0.00031
FGB	Fibrinogen beta chain	-0.86	<0.0001
APLP2	Amyloid beta precursor like protein 2	-0.73	0.001
SLC25A5	ADP/ATP translocase 2	-0.71	<0.0001
PHB2	Prohibitin	-0.7	<0.0001
COX4I1	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	-0.68	0.001
PHB	Prohibitin-2	-0.67	<0.0001
ANXA2	Annexin A2	-0.65	<0.0001
FGA	Fibrinogen alpha chain	-0.65	<0.0001
HIST1H2AC	Histone H2A	-0.64	0.001
ANXA4	Annexin A4	-0.61	0.00039
ANXA3	Annexin A3	-0.59	0.00016
RPN1	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 1	-0.59	0.00051
HIST1H4	Histone H4	-0.55	0.001
IMMT	MICOS complex subunit MIC60	-0.54	0.00015
ANXA11	Isoform 2 of Annexin A11	-0.53	0.001
H2AFY	Core histone macro-H2A	-0.53	<0.0001
C3	Complement C3	-0.49	<0.0001
RAP1B	Ras-related protein Rap-1b	-0.47	0.001
APOA1	Apolipoprotein A-I	-0.46	0.001
HIST1H1E	Histone cluster 1 H1 family member e	-0.42	0.0001
EZR	Ezrin	-0.42	<0.0001
A2M	Alpha-2-macroglobulin	-0.36	0.001
ANPEP	Aminopeptidase N	-0.27	<0.0001

exposure, and their interaction included in the model. For mass spectrometry, peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Normalization was performed iteratively (across samples and spectra) on intensities, as described in [15]. Medians were used for averaging. Spectra were log transformed, pruned of those matched to multiple proteins, and weighted by adaptive intensity weighting logarithm. The FDR was adjusted using Benjamini–Hochberg procedure ($P < 0.05$) to identify significance based on permutation tests.

Pathway analysis

Identification of enriched pathways was conducted using Ingenuity Pathway Analysis (IPA; Qiagen) software. A list of DEGs or differentially expressed proteins (DEPs) was uploaded to the IPA tool. IPA functional analysis tools identified biological functions and/or pathways that were most significant to the data set ($P < 0.05$) according to a righted tailed Fisher exact test. IPA currently supports only human, mouse, and rat species with full content, so the bovine species is supported at the ortholog level; therefore some significant genes and proteins may not be included in the analysis. Emphasis was given to top canonical pathways and molecular and cellular functions.

Results

Endometrium gene expression

There were 17,765 genes identified in the current study. The results and discussion focus on differentially expressed genes (DEGs) based on preovulatory estradiol exposure (highE2 versus lowE2 animals), and the interaction between preovulatory estradiol exposure and conceptus presence (highE2/conceptus versus lowE2/conceptus).

Preovulatory estradiol exposure (highE2 versus lowE2). There were 1,111 DEGs between the highE2 and lowE2 groups. Specifically, there were 619 genes that were downregulated (*KRT1, VGF, OXTR, CLDN10, NPY*) and 492 genes that were upregulated (*IDO1, CXCL10, AQP8, KRT17, OAS2*) in the highE2 group. Top canonical pathways associated with these DEGs included: interferon signaling (2.32E-07), activation of interferon regulatory factors by cytosolic pattern recognition receptors (8.39E-06), hepatic fibrosis/hepatic stellate cell activation (7.31E-05), retinoic acid-mediated apoptosis signaling (1.88E-04), and agranulocyte adhesion and diapedesis (9.52E-04). The main molecular and cellular functions associated with DEGs were: cell cycle (182 molecules; 2.20E-05-5.65E-17), cellular movement (224 molecules; 3.23E-05-4.23E-15), cellular development (204 molecules; 2.18E-05-3.57E-13), cellular growth and

Table 5. DEPs upregulated in highE2/conceptus animals compared with lowE2/conceptus animals.

Abbreviation	Protein name	Log2Fold change	FDR
PLEC	Plectin	0.2	<0.0001
ATP5B	ATP synthase subunit beta, mitochondrial	0.25	0.001
HSPD1	60 kDa heat shock protein, mitochondrial	0.26	0.00068
IDH2	Isocitrate dehydrogenase [NADP], mitochondrial	0.26	0.001
CNDP2	Cytosolic nonspecific dipeptidase	0.29	0.00039
ALDH18A1	Delta-1-pyrroline-5-carboxylate synthase	0.3	0.001
HNRNPK	Heterogeneous nuclear ribonucleoprotein K	0.32	0.001
ACTN4	Alpha-actinin-4	0.32	<0.0001
IDH1	Isocitrate dehydrogenase [NADP]	0.33	0.00061
PRKAR2A	cAMP-dependent protein kinase type II-alpha regulatory subunit	0.37	0.00042
SPTBN1	Spectrin beta chain	0.38	<0.0001
GPD1L	Glycerol-3-phosphate dehydrogenase [NAD(+)]	0.39	0.001
ALDH2	Aldehyde dehydrogenase, mitochondrial	0.4	0.00049
HSD17B4	Hydroxysteroid (17-beta) dehydrogenase 4	0.4	<0.0001
GPLD1	Phosphatidylinositol-glycan-specific phospholipase D	0.56	<0.0001
ACAA1	Acetyl-CoA acyltransferase 1	0.58	0.00067
TST	Thiosulfate sulfurtransferase	0.58	<0.0001
GSTM4	Glutathione S-transferase Mu 1	0.66	0.001
GOT1	Aspartate aminotransferase, cytoplasmic	1.02	<0.0001

proliferation (237 molecules; 2.75E-05-3.57E-13), and cell death and survival (273 molecules; 2.59E-05-4.03E-13).

Preovulatory estradiol and conceptus presence interaction. There were 432 DEGs between the highE2/conceptus and the lowE2/conceptus groups. Specifically, there were 253 genes that were downregulated (*UTMP, C3, MYL9, ADAM12*, and *LPL*) in the highE2/conceptus (Figure 1). There were 179 genes that were upregulated (*PRSS8, MUC13, IDO1 CXCL10, CXCL11*, and *FBP1*) in the highE2/conceptus group compared with the lowE2/conceptus group (Figure 1). Table 3 emphasizes the importance of select DEGs in various biological processes. Top canonical pathways associated with these DEGs included: calcium signaling (7.73E-05), caveolar-mediated endocytosis signaling (1.00E-04), agranulocyte adhesion and diapedesis (1.77E-04), and axonal guidance signaling (2.37E-04). The main molecular and cellular functions associated with the DEGs were: cellular movement (97 molecules; 2.21E-03–9.63E-08), molecular transport (81 molecules; 1.87E-03–2.68E-07), cellular growth and proliferation (79 molecules; 2.02E-03–4.80E-07), cellular assembly and organization (74 molecules; 1.45E-03–1.24E-05), and cell death and survival (124 molecules; 2.22E-03–2.18E-05).

RT-PCR endometrium validation. In endometrium, highE2 animals had increased mRNA abundances of *PRSS8* ($P = 0.005$; Figure 2), *CXCL10* ($P = 0.003$; Figure 2), *IDO1* ($P = 0.02$; Figure 3), *FABP3* ($P = 0.04$; Figure 3), *XAF1* ($P = 0.05$; Figure 5), and *DDX58* ($P = 0.007$; Figure 5) on d16. HighE2 animals had decreased *OXTR* mRNA abundance ($P = 0.02$; Figure 4) in the endometrium compared with lowE2 animals. HighE2 animals also had a tendency to have increased mRNA abundance of *MUC13* ($P = 0.07$; Figure 3) compared with animals with low preovulatory estradiol concentrations. There was no difference in *CLDN4* ($P = 0.26$; Figure 2), *RSAD2* ($P = 0.17$; Figure 4), *ISG15* ($P = 0.85$; Figure 4), and *PARP12* ($P = 0.23$; Figure 5) mRNA abundance in the endometrium among highE2 and lowE2 animals. There was a conceptus and preovulatory exposure interaction for *CXCL10* ($P = 0.04$; Figure 6) and a tendency for an interaction for *IDO1* ($P = 0.06$; Figure 7) and *DDX58* ($P = 0.06$; Figure 8).

TE gene expression

There were no differences in mRNA abundances for *IFNT, PTGS2, TM4SF1, C3*, and *FGFR2* in the TE collected from highE2 and lowE2 animals on d16 of pregnancy ($P > 0.22$).

Uterine protein expression

Proteins in ULF were identified and quantified using mass spectrometry. This approach detected 6989 peptides and 1269 proteins in the pools of ULF. The results and discussion focus on differentially expressed proteins based on preovulatory estradiol exposure (highE2 versus lowE2) among animals that had a conceptus recovered from their reproductive tract (highE2/conceptus versus lowE2/conceptus).

HighE2/conceptus versus lowE2/conceptus. There were 48 DEPs between the highE2/conceptus and the lowE2/conceptus groups. Specifically, there were 29 proteins that were downregulated (*UTMP, ORM1, ANXA8, ANXA1*, and *FGG*; Table 4) and 19 proteins that were upregulated (*GOT1, GSTM4, TST, ACAA1*, and *GPLD1*) in the highE2/conceptus group compared with the lowE2/conceptus group (Table 5). Top canonical pathways associated with these DEPs included: acute phase response signaling (3.17E-05), aryl hydrocarbon receptor signaling (5.06E-04), coagulation system (6.39E-04), and PPAR signaling (9.87E-04). The main molecular and cellular functions associated with the DEPs were carbohydrate metabolism (four molecules; 3.99E-02–1.10E-06), nucleic acid metabolism (eight molecules; 4.60E-02–3.29E-06), small molecule biochemistry

(14 molecules; 4.60E-02–3.29E-06), cellular development (4.01E-02–9.92E-05), and cellular function and maintenance (12 molecules; 4.91E-02–9.92E-05).

Discussion

Transcription in the endometrium is mainly regulated by complex interactions of estradiol and progesterone. These hormones act as transcription factors by binding to nuclear receptors causing conformational changes, ultimately allowing receptors to bind to chromatin and cause transcriptional changes within hours [16, 17]. Previous researchers have focused on differences in transcriptomes [18–22] and proteomes [21, 23–26] during various time points in the preimplantation phase of development in ruminants. However, prior to the current study there was little known about the impact of preovulatory estradiol on uterine and TE transcripts around the critical period of maternal recognition of pregnancy.

There has been extensive research regarding the impact of preovulatory estradiol on the uterine environment and embryo survival in cattle. On d6, heifers that exhibited estrus yielded embryos that were more advanced in stage and had improved quality when compared with heifers that did not exhibit estrus; however, recovery rates were not different [11]. Bridges et al. [27] concluded that there was no difference in conceptus size and interferon tau concentrations on d15.5 based on preovulatory estradiol exposure. On d16, Northrop et al. [10] determined that there were no differences in conceptus survival based on apoptosis in the TE, IFNT, protein, and glucose concentrations in ULF among highE2 and lowE2 animals. On day 19, Davoodi and colleagues reported that genes associated with maternal immune system, attachment between the endometrium and conceptus, and Corpus Luteum (CL) maintenance were favorably expressed in cows that exhibited estrus near the time of AI compared with cows that did not. Additionally, they reported that cows that exhibited estrus yielded longer conceptuses [28]. The following discussion focuses on biological processes associated with the DEGs and DEPs among highE2 and lowE2 animals on d16 of pregnancy in cattle.

Forde et al. [24] previously analyzed protein content in ULF from cyclic and pregnant heifers on d16. They further analyzed proteins specifically produced by day 16 conceptuses in culture media. Thirty proteins were identified to be unique to ULF from pregnant heifers and produced by short-term in vitro cultured conceptuses on d16 [24]. In the current study, 28 of these proteins were identified on d16 in ULF. There were 20 proteins that were upregulated, and 8 proteins that were downregulated in the highE2/conceptus group compared with the lowE2/conceptus group. They concluded that these proteins could possibly be involved in facilitating interactions between the conceptus and endometrium during pregnancy recognition. If this is true, these proteins may contribute to the improved pregnancy rates observed in cows that display estrus during a timed AI protocol [9, 29].

Endometrial remodeling

Remodeling of the endometrium and chorionic extracellular matrix (ECM) is critical for successful implantation and placentation. Specifically, remodeling of the ECM supports proliferation, differentiation, migration of binucleate cells, and attachment [30]. Other studies in ruminants have reported genes involved in this process being expressed during gestation [17, 31]. RNA sequencing revealed that *MYLK, ADAM12, TAGLN, ACTA2, MYL9, COL1A2, COL3A1, HSPE,* and *ADAMTS15* were downregulated genes, whereas *PRSS8, LGMN*, and *GRN* were upregulated in the endometrium of highE2 animals with a conceptus compared with lowE2 animals with a conceptus. Specifically, *PRSS8* is a serine protease that is reported to play a role in endometrial epithelial morphology establishment, tissue remodeling, and trophoblast invasion during early pregnancy in the rhesus monkey [32]. Additionally, *PRSS8* knockout mice exhibited lethality due to placental insufficiency [33]. The validation of *PRSS8* by RT-PCR suggests that these remodeling pathways are contributing to increased pregnancy success in cows that exhibit behavioral estrus.

Adhesion

Adhesion molecules play a critical role in the attachment process between fetal chorionic binucleate cells and luminal epithelium. In the current study, RNA sequencing revealed that *CLDN4, F5, MUC13, ITGA3, CLEC4F, ITGB5* genes were upregulated, whereas *CDH4* and *TROAP* were downregulated when comparing the highE2/conceptus and lowE2/conceptus groups. Mucins are heavily glycosylated proteins that contain glycans that are recognized by blastocyst [34]. Specifically, *MUC13* mRNA was upregulated in pregnant caruncular tissue on d20 of pregnancy in cattle [19]. Additionally, *MUC13* mRNA was increased in the endometrium of highly fertile and subfertile heifers compared with infertile heifers on d17 of gestation [22]. Forde et al. [35] reported increased *MUC13* expression in the endometrium as pregnancy progressed (d13 to d19). In the current study, RT-PCR validation determined that *MUC13* had a tendency to be increased in highE2 endometrium compared with lowE2 endometrium.

In the present study, mass spectrometry also identified that actinin 4 (*ACTN4*) had increased abundance in ULF among the highE2/conceptus group. Previously, on d16 in sheep, around time of attachment, *ACTN4* was elevated in pregnant ULF compared with nonpregnant ULF [23]. The alpha actinin family proteins are involved in growth and remodeling. They can bind to filamentous actin and regulate cytokinesis, cell adhesion, spreading, migration, and signaling [36]. An increase in expression of cell adhesion molecules at the mRNA and protein level among highE2 pregnant animals suggests that the endometrium is undergoing changes in order to prepare for attachment prior to d19, which may increase the likelihood of survival to d29 among these animals.

Immune

The developing conceptus is made up of both maternal and paternal genes. The innate and adaptive immune system must be appropriately regulated to prevent rejection of the conceptus during pregnancy, as it is considered foreign by the female body. For RNA sequencing, the following genes associated with immune response were increased when comparing highE2/conceptus versus lowE2/conceptus: *IDO1, BPI, BOLA-NC1, C2, CFB, C1QL2, OAS2, S100A12, CD48, MIC1, CXCL10,* and *CXCL11*. RT-PCR results with additional animals added further confirmed sequencing data that there was an effect of preovulatory estradiol, conceptus presence, and an interaction on *IDO1* and *CXCL10* transcript abundance in the endometrium on d16 in the present study. Indoleamine 2, 3-dioxygenase (*IDO1*) catalyzes the rate-limiting step in tryptophan catabolism. Previous research determined that placental cells express *IDO*, which causes tryptophan depletion leading to suppression of T-cell proliferation at the maternal–fetal interface [37]. In pregnant mice, treatment with an IDO inhibitor

resulted in inhibition of tryptophan catabolism, which initiated maternal lymphocytes to facilitate fetal rejection [37].

Chemokines are multifunctional molecules that recruit immune cells to inflammatory regions [38]. Specifically, there is abundant chemokine expression at the maternal–fetal interface. In humans, leukocytes are infiltrated to the implantation site, and they are responsible for maintaining an appropriate balance between embryo protection and accepting hemiallogenic tissues [39]. Specifically, on d17 there was more than an 11-fold increase in CXCL10 expression in the uterus of pregnant cows [40]. *CXCL10* mRNA expression was also downregulated in subfertile dairy cows compared with fertile cows on d17 of pregnancy [41]. On d19, *CXCL10* mRNA expression in the uterus was favorably expressed on d19 of gestation among cows that exhibited estrus around the time of AI compared with animals that did not [28].

Additionally, mass spectrometry revealed increased *HSP60* protein abundance among highE2/conceptus animals compared with lowE2/conceptus animals, whereas uterine milk protein (*UTMP*) and complement 3 protein (*C3*) had decreased protein abundance in the highE2/conceptus group. Specifically, the complement component system functions in both embryonic and host protection [42]. Furthermore, an intact complement system during early pregnancy at the placental interface optimizes placental development and function [43]. In humans, previous research has established that excessive or misdirected complement activation can lead to pregnancy complications such as pregnancy loss, fetal growth restriction, and preterm birth [43]. Therefore, the uterus and/or conceptus in highE2 animals may be downregulating the abundance of this protein to avoid pregnancy loss. Together these data confirm the importance of uterine immune function in recognition of pregnancy and suggest that function of this pathway differs between females with increased circulating E2 concentrations at estrus compared with females with decreased circulating E2 concentrations at estrus.

Metabolic

An increase in metabolites during pregnancy is necessary to ensure adequate nutrients for growth, development, and survival of the conceptus. The following genes associated with metabolic function were upregulated: *FABP3, SLC2A1, SLC5A5, SLC27A2, SLC27A5, AMPD3, FBP1*, and ACO2, whereas *LPL, SLC7A2, SLC38A4*, and *FAAH* were downregulated when comparing highE2/conceptus and lowE2/conceptus. Mass spectrometry revealed that the following proteins/enzymes associated with metabolic function were upregulated: *ACAA1, IDH1, IDH2, GPD1L*, and *GOT1*.

Lipids are essential for structural properties, providing energy for proliferating tissue, cell signaling, and generation of ATP. Endometrium is the main source of lipids, which are especially important during rapid conceptus elongation in ruminants. Fatty acid-binding protein (*FABP3*) is involved in uptake, metabolism, and transport of long chain fatty acids. On d17, *FABP3* mRNA in the endometrium was increased among high-fertility heifers compared with subfertile heifers [22]. It also had increased expression in the caruncular tissue of pregnant animals compared with cyclic animals on d20 of gestation [19]. In the current study, RT-PCR results with additional animals added further confirmed sequencing results that there was an effect of preovulatory estradiol on *FABP3* transcript abundance in the endometrium on d16. However, there was no preovulatory estradiol and conceptus interaction.

Glucose is one of the main energy sources used by conceptuses for growth and development, it is known to regulate trophoblast proliferation and function [44]. Transport of glucose into the uterus is mediated by facilitative and/or sodium-dependent transporters. The *SLC2A1* transporter has been localized mainly in the glandular and luminal epithelial cells [45]. This glucose transporter appears to be regulated by both progesterone and interferon tau in the glandular epithelium [46]. Previously, our laboratory reported that highE2 animals had increased *SLC2A1* mRNA abundance in intercaruncular and caruncular tissue compared with lowE2 animals on d16 [10].

RNA sequencing and mass spectrometry similarities

There were six DEPs (*ANXA8, APLP2, PHB, ANPEP, ALDH2, GPLD1*) that were also considered differentially expressed (FDR < 0.10) at the mRNA level. Lack of similarities in mRNA and protein expression may be attributed to: posttranscriptional (alternative splicing, transport, mRNA stability), translational (miRNAs), and posttranslational (phosphorylation, ubiquitination, methylation, and acetylation) regulatory factors. Similar pathways associated with DEGs and proteins among highE2 and lowE2 animals with a conceptus included: calcium signaling, protein kinase A signaling, corticotropin-releasing hormone (CRH) signaling, and LPS/IL-1-mediated inhibition of RXR function. Calcium signaling in humans has been reported to be crucial for implantation and placental development [47]. Additional functions include: second messenger in signal transduction pathways, motility, apoptosis, regulation of mitochondrial function, and cell cycle progression [48]. Protein kinase A signaling functions in regulating metabolism and cell growth/proliferation. CRH is involved in anti-inflammatory response, stromal cell decidualization in humans [49], and implantation in mice [50]. The LPS/IL-1-mediated inhibition of RXR function pathway ultimately causes release of proinflammatory cytokines (IL-1) leading to a decrease in expression of hepatic genes that leads to impaired metabolic activity [51].

In summary, this study identified differences in critical genes/proteins and pathways among highE2 and lowE2 animals on day 16 of pregnancy. These differences in uterine function, specifically relating to metabolism, immune regulation, endometrial remodeling, and adhesion may be preparing the conceptus for improved likelihood of survival after d16 among highE2 animals. We hypothesize that these differences in conceptus survival are most likely occurring around the time of attachment; however, further research is needed to determine underlying biological mechanisms that lead to increased conceptus survival among highE2 animals between d16 and d29.

Disclosure statement

Names are necessary to report factually on available data; however, USDA neither guarantees nor warrants the standard of products, and use of names by USDA implies no approval of the product to the exclusion of others that may also be suitable. USDA is an equal opportunity provider and employer.

Author contribution

E.J.N.: this project was part of her dissertation. She helped design the study, collected the data, analyzed the data, and wrote the manuscript.
J.J.J.R.: helped with the collection of all the data.
R.A.C.: helped with the design of the study, collection of the data, analysis of the data, and writing of the manuscript.
R.Y.: helped with the analysis of the data.

X.G.: helped with the analysis of the data.
G.A.P.: this study was conducted in his laboratory. He helped with the design of the study, the collection of the data, analysis of the data, and writing of the manuscript.
Conflict of interest: The authors have declared that no conflict of interest exists.

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