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Sexually Dimorphic Gene Expression in Bovine Conceptuses at the Initiation of Implantation[1]

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ABSTRACT

In cattle, maternal recognition of pregnancy occurs on Day 16 via secretion of interferon tau (IFNT) by the conceptus. The endometrium can distinguish between embryos with different developmental competencies. In eutherian mammals, X-chromosome inactivation (XCI) is required to ensure an equal transcriptional level of most X-linked genes for both male and female embryos in adult tissues, but this process is markedly different in cattle than mice. We examined how sexual dimorphism affected conceptus transcript abundance and amino acid composition as well as the endometrial transcriptome during the peri-implantation period of pregnancy. Of the 5132 genes that were differentially expressed on Day 19 in male compared to female conceptuses, 2.7% were located on the X chromosome. Concentrations of specific amino acids were higher in the uterine luminal fluid of male compared to female conceptuses, while female conceptuses had higher transcript abundance of specific amino acid transporters (*SLC6A19* and *SLC1A35*). Of note, the endometrial transcriptome was not different in cattle gestating a male or a female conceptus. These data support the hypothesis that, far from being a blastocyst-specific phenomenon, XCI is incomplete before and during implantation in cattle. Despite differences in transcript abundance and amino acid utilization in male versus female conceptuses, the sex of the conceptus itself does not elicit a different transcriptomic response in the endometrium.

amino acids, endometrium, gene expression, uterine luminal fluid, XCI

INTRODUCTION

In cattle, pregnancy recognition both at the physiological [1, 2] and transcriptomic level [3, 4] is initiated on Day 16 in order to prevent release of luteolytic pulses of prostaglandin F2 alpha from the endometrium, corpus luteum regression, and subsequent return to cyclicity. Subtle responses of the endometrium to the conceptus can be detected as early as Day 13 [5], but the major transcriptomic response to the pregnancy recognition signal in cattle, interferon tau (IFNT) from the cells of the conceptus trophectoderm, occurs at Day 16 [3]. Additional studies have demonstrated a high degree of similarity in the changes in the endometrial transcript abundance between pregnant and cyclic endometrium as pregnancy progresses from Day 15 through Day 20, that is, during the period of maternal recognition of pregnancy [3, 4, 6–9]. The endometrial transcriptomic response to early pregnancy is quite specific to the type of conceptus present, such that by Days 18 and 20 the response signature can distinguish between in vivo, in vitro, and cloned embryos and thus the developmental outcome of the embryo [10, 11].

In eutherian mammals, X-chromosome inactivation (XCI) is required in females to ensure an equal transcriptional level of most X-linked genes for both males and females. In female (XX) preimplantation embryos, both X chromosomes are transcriptionally active from embryonic genome activation until a long noncoding RNA (X-inactive specific transcript: *XIST*) mediates the inactivation of one of them. Okamoto et al. [12] revealed substantial diversity in the timing and regulation of XCI initiation between mice, in which this phenomenon has been studied in most detail, rabbits, and humans. For example, *XIST* transcript abundance is not imprinted in rabbit and human embryos, and the choice of which X chromosome to inactivate seems to occur downstream of *XIST* upregulation and X-chromosome coating, which differs significantly from the processes in the mouse.

We found that the process of XCI in the bovine differs markedly from that of the mouse. Similar to the situation reported in humans, XCI in cattle is far from being accomplished at the blastocyst stage [13]. Furthermore, abundance of many X-linked transcripts that escaped XCI in the bovine blastocyst were effectively equalized among sexes in Day 14 elongated conceptuses [14]. This mirrors the situation in the rabbit late blastocyst and suggests that a large component of XCI occurs after the differentiation of trophectoderm/inner cell mass lineages, but before gastrulation, indicating a significant amount of discord between the mouse

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and many other mammalian species. In addition, we found sexually dimorphic differences exist between male and female embryos in terms of developmental rate [15], and Day 7 blastocyst transcriptome [13] as well as amino acid turnover [16], with as many as one-third of all actively expressed transcripts in the blastocyst being determined by the sex of the embryo [13].

In this study, we hypothesized that sex-related differences in transcript abundance remain throughout conceptus elongation and that male and female embryos elicit a different response in the endometrium. Thus, the aims of this study were to 1) examine the effect of conceptus sex on conceptus transcript abundance and amino acid utilization at Day 19, 2) compare the temporal changes in conceptus transcript abundance between Days 7 and 19, and 3) determine whether male and female embryos on Day 19 elicit a different response from the endometrium.

MATERIALS AND METHODS

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1876) and the European Community Directive 86/609/EC and were sanctioned by the Animal Research Ethics Committee of University College Dublin. Unless otherwise stated, all chemicals and reagents were sourced from Sigma.

Animal Model and Sample Collection

The estrous cycles of crossbred beef heifers (n = 30) were synchronized using an 8-day controlled internal drug release device (1.38 g progesterone; Pfizer Animal Health) placed intravaginally. One day prior to controlled internal drug release device removal, a 2 ml intramuscular injection of a prostaglandin $F_{2\alpha}$ analog (Estrumate, equivalent to 0.5 mg cloprostenol; Intervet) was administered. All heifers were then observed at 4 h intervals, and only those observed in standing estrus (equals Day 0) were inseminated with semen from a proven sire. All heifers were slaughtered on Day 19 following estrus corresponding to the initiation of implantation in cattle. Thirty minutes after slaughter, each uterine horn was flushed with 10 ml of PBS, and the presence of a conceptus was observed under a stereo-microscope. Only those heifers from which a conceptus was recovered were further processed for tissue collection (n = 24). Each conceptus was dissected into four pieces, three containing only extra-embryonic tissue (EET) and one containing the embryonic disk along with associated trophectoderm cells, and immediately snap-frozen in liquid nitrogen. The uterine luminal flush samples were then placed into 1 ml aliquots and snap frozen in liquid nitrogen prior to amino acid analysis. The uterine horn ipsilateral to the corpus luteum was opened longitudinally, and intercaruncular endometrium from the midpart of the horn was dissected away from the underlying myometrium and snap frozen in liquid nitrogen. All samples were stored at −80°C prior to processing.

Conceptus Sexing

DNA was extracted from a sample of EET cells from each conceptus with phenol/chloroform treatment and finally resuspended in 200 μl of Milli-Q water. Two microliters of each sample were used to perform embryo sexing by PCR amplification of sex-specific polymorphic fragments in the amelogenin gene as previously described [17].

RNA Extraction and Microarray Hybridization

Total RNA was extracted from the EET cells from confirmed female (n = 5) and male (n = 5) conceptuses as well as their corresponding intercaruncular endometrial tissue (100 mg) using Trizol reagent as per manufacturer's instructions (Invitrogen). Following on-column DNase digestion and RNA cleanup, (Qiagen), both the quality and quantity of the RNA was determined using an Agilent Bioanalyzer (Agilent Technologies) and a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc.), respectively. A subset of samples (both conceptus and corresponding intercaruncular endometrial tissue) with an RNA integrity number of greater than 8.0 were randomly chosen for microarray analysis (n = 5 per tissue type). Transcriptomic analysis was carried out using the Affymetrix GeneChip Bovine Genome Array. Two micrograms of total RNA were converted to cDNA via first and second strand synthesis using the GeneChip Expression 3′-Amplification One-Cycle cDNA Synthesis kit. Biotin-labeled cRNA was synthesized from double-stranded cDNA using the GeneChip Expression 3′-Amplification In Vitro Transcription Labeling kit. Complementary RNA quality was assessed on the Agilent 2100 Bioanalyzer, and 25 μg of cRNA was fragmented using 5× fragmentation buffer in RNase-free water contained within the GeneChip Sample Cleanup Module at 94°C for 35 min and quality accessed again on the Agilent 2100 Bioanalyzer. Fifteen micrograms of fragmented cRNA and hybridization cocktail were added to the GeneChip Bovine Genome Array and hybridized for 16 h at 45°C. Each array was then washed and stained on the GeneChip Fluidics Station 450 using the appropriate fluidics script, and once completed, the array was inserted into the Affymetrix autoloader carousel and scanned using the GeneChip Scanner 3000.

The raw signal intensities were read into R and preprocessed using functions of both affy and GCRMA packages of the BioConductor project [18]. Hierarchical clustering analysis was performed to determine the greatest source of variation in the tissue samples. Lists of differentially expressed genes (DEGs) were determined by the Limma package [19] employing linear modeling and an empirical Bayes framework to shrink the variance of measurements on each probe set. A modified *t*-test was then carried out, and all *P* values were adjusted for multiple testing using the Benjamini and Hochberg false discovery rate method.

Analysis of IFNT in the Uterine Luminal Fluid

Concentrations of IFNT in the uterine luminal fluid (ULF) recovered from heifers with a male (n = 11) or female (n = 11) conceptus was carried out using a cytopathic antiviral assay [20, 21] as previously reported [22]. Samples were examined in duplicate by titrating in a 96-well plate (1:3 serial dilutions). Madin-Darby bovine kidney cells were added and incubated in medium (Dulbecco modified Eagle medium containing 10% fetal bovine serum) at 37°C in 5% CO_2 in humidified air. After 24 h, the cells were challenged with vesicular stomatitis virus for 1 h. Thereafter, virus was removed, and cells were incubated with growth medium (Dulbecco modified Eagle medium containing 10% fetal bovine serum) for 18 h. Cell viability (75%) was determined after fixation using 0.5% (w/v) gentain-violet. The ability of samples to prevent lysis by 50% was compared with a recombinant human IFN alpha standard (3.8×10^8 IU/mg; EMD Biosciences). Data are presented as international units of antiviral activity per ml of conditioned medium. Unconditioned medium (blanks) did not contain antiviral activity.

Analysis of Amino Acid Content of ULF

The amino acid composition of ULF (n = 11 samples hosting a male and n = 11 samples hosting a female conceptus) was quantitatively analyzed by high-performance liquid chromatography as previously described [16]. Briefly, amino acids in ULF were derivatized with o-phthaldialdehyde reagent, supplemented with 1 mg/ml 2-mercaptoethanol. Derivatized samples were subjected to reverse phase chromatography using an Agilent 1100 Series high-performance liquid chromatography system coupled with a Phenomenex HyperClone 5 mm C-18 ODS 250 × 4.6 mm column. A gradient elution with two buffers—(A) 80% 83 mM sodium acetate, 19.5% methanol, and 0.5% tetrahydrofuran, and (B) 80% methanol and 20% 83 mM sodium acetate—was used to separate o-phthaldialdehyde-amino acid conjugates at 30°C with a flow rate of 1.3 ml/min for 60 min per sample. Concentrations of amino acids in the ULF (in micromolar) were determined by comparing sample peak areas to those from certified standards.

Quantitative Real-Time PCR Analysis

One thousand nanograms of total RNA from the conceptus and corresponding endometrial tissue of five male and five female conceptuses were subjected to reverse transcription reaction using Superscript III (Applied Biosystems) and random hexamers as per the manufacturer's instructions. Primers for microarray validation were designed using Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon-exon boundaries where possible. Primers for amino acid transporters have been previously reported [23]. Each quantitative real-time PCR (qRT-PCR) reaction was carried out on the 7500 Fast Real-Time PCR System (Applied Biosystems) with 5 ng of cDNA, optimized primer concentrations (Supplemental Table S1; all Supplemental data are available online at www.biolreprod.org), and 7.5 μl FAST Sybergreen mastermix (Applied Biosystems) in a final reaction volume of 15 μl. The cycling conditions for all qRT-PCR reactions were as 2 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. All reactions were carried out in duplicate, with the inclusion of a dissociation curve to ensure specificity of amplification as well as no template controls. A standard curve was included for each gene of interest as well as for the

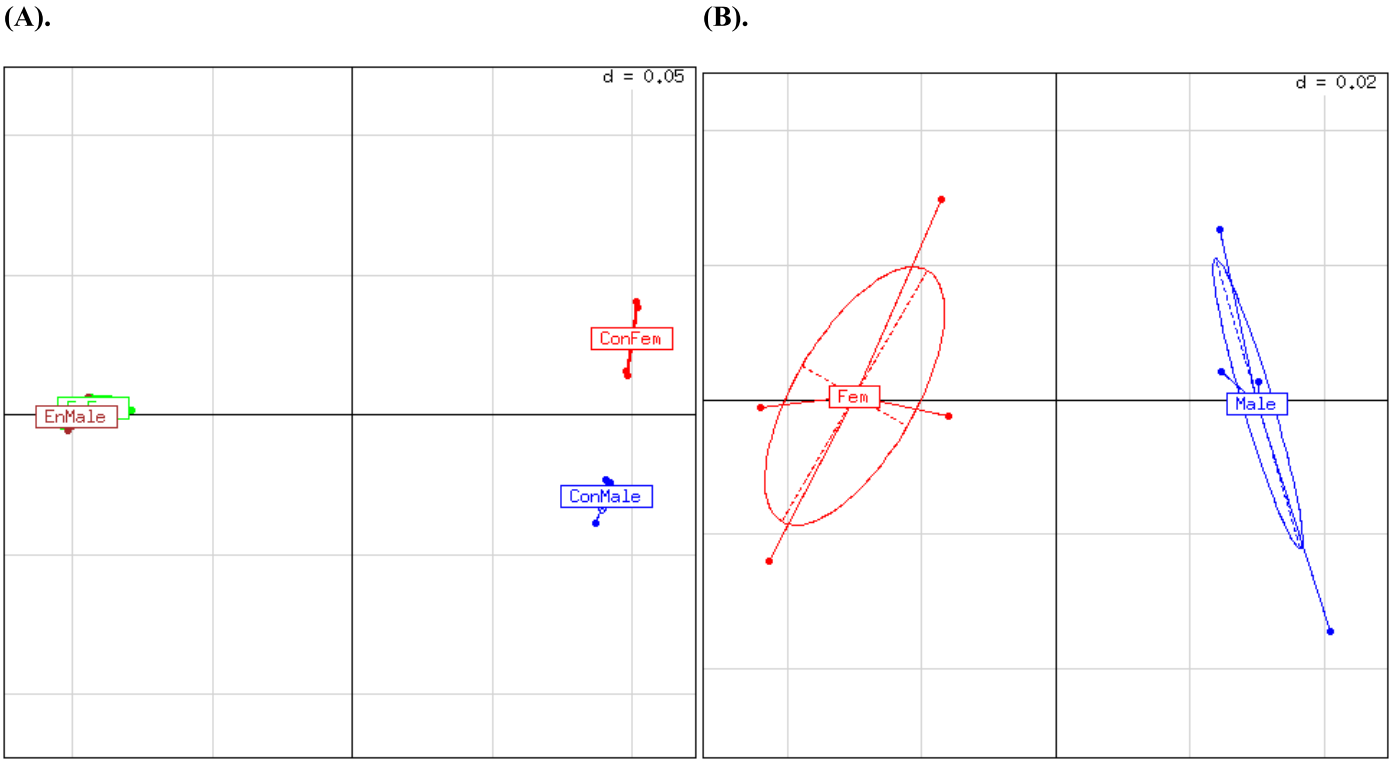

FIG. 1. Correspondence analysis indicating the source of greatest variation in the overall transcriptional profile. Each dot represents an individual microarray for an individual sample. **A**) A clear segregation in the expression profiles of the different tissue types, that is, endometrium and conceptus, was observed, but sex of the conceptus affected gene expression in the conceptus (right) but not the endometrium (left). **B**) Differences in overall gene expression profiles in male and female conceptus when plotted alone.

normalizer gene to obtain primer efficiencies. All raw cycle threshold values were then imported into qBasePlus software (Biogazelle) where data were calibrated, normalized, and expression values for each gene were determined in arbitrary units, that is, calibrated normalized relative quantities (CNRQ).

Overrepresented Gene Ontology Terms, Upstream Regulators, and Pathway Analysis

To further interrogate the differences in transcript abundance associated with the sex of the conceptus, the list of DEGs was subjected to analysis using the functional annotation tool in DAVID (http://david.abcc.ncifcrf.gov/) to generate overrepresented biological processes and molecular functions. In addition, Ingenuity Pathway Analysis (IPA) was performed (http://www.ingenuity.com) to identify overrepresented pathways associated with the sex of the conceptus, that is, those with a larger number of DEGs that one would expect by chance. IPA was also used to assess whether or not the sex of the conceptus modified upstream regulators of the DEGs identified between male and female conceptuses. For all overrepresented gene ontologies, pathways, and upstream regulators a *P*-value for a given function was calculated by considering the number of functional analysis molecules that participate in that function, for example, ligand or receptor, and the total number of molecules that are known to be associated with that function/pathway in the DAVID/Ingenuity Knowledge Base. Gene ontology terms, pathways, and/or upstream regulators with *P*-values less than 0.05 were considered significant (i.e., more DEGs associated with these than would be expected by chance).

RESULTS

Correspondence analysis revealed segregation between the different tissue types (i.e., endometrial tissue data clustered together and conceptus tissue data clustered together) (Fig. 1A). Conceptus sex did not affect the overall transcriptional profile of the endometrium but did affect its global transcript abundance in the conceptus (Fig. 1B).

Differential Transcript Abundance in Day 19 Male and Female Conceptuses

In Day 19 conceptuses, the abundance of 5132 transcripts were significantly different between males and females ($P < 0.05$); 2498 were increased in male compared to female conceptuses while 2634 genes were increased in female compared to male conceptuses. Full gene descriptions, associated *P*-values, and log2 fold change differences are given in Supplemental Table S2. Of the transcripts whose abundance was highest in male conceptuses to the greatest extent, zinc finger protein 665-like (2.63 log2 fold change increase in male), lumican (2.34), zinc finger protein 107 (2.33), mirror-image polydactyly gene 1 protein-like (2.20), micro-RNA mir-2284d (2.03), unknown gene (2.01), zinc finger protein 208 (1.95), micro-RNA mir-2399 (1.95), CDC-like kinase 1 (1.91), and zinc finger protein 91-like (1.87), none were located on the X chromosome. Transcripts whose abundance was increased in female conceptuses compared to male to the greatest extent included XIST (4.65 log2 fold increase in female compared to male conceptuses), immunoglobulin light chain VJ region (2.44), intestine-specific transcript 1 protein (2.17), synapsin I (1.91), uncharacterized protein C12orf54 homolog (1.84), TIMP metallopeptidase inhibitor 1 (1.69), pancreatic progenitor cell differentiation and proliferation factor homolog (zebrafish) (1.5), KxDL motif containing 1 (1.48), histone cluster 1, H3a-like 23 (1.47), and mucin 15, cell surface associated (1.45). Of these, X (inactive)-specific transcript, synapsin I, and TIMP metallopeptidase inhibitor 1 are located on the X chromosome. Of the total number of DEGs identified between male and female conceptuses, 140 were located on the X chromosome and 78 were increased in the female conceptuses while 62 were

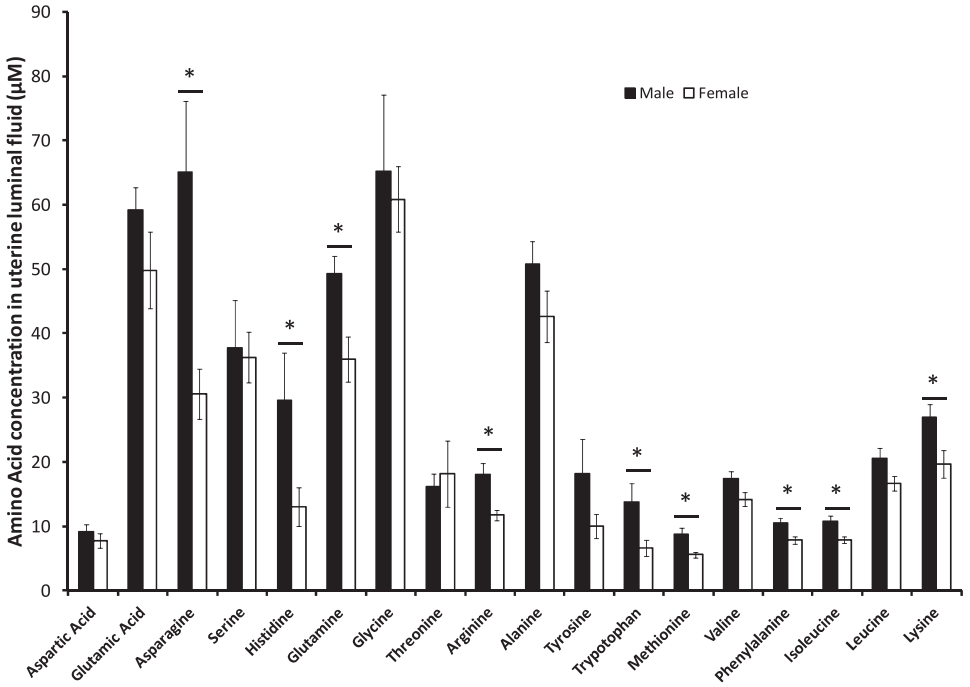

FIG. 2. Concentrations (μM ± SEM) of amino acids in the uterine luminal fluid (ULF) recovered on Day 19 of pregnancy from heifers with a male (black bars) or female (open bars) conceptus present (n = 11 per sex). Significant differences in amino acid concentrations from the uterus containing a male versus a female conceptus are noted with an asterisk (*) when $P < 0.05$.

increased in the male conceptus on Day 19 of pregnancy. No probes on the microarray were located on the Y chromosome.

Gene Ontology and IPA of Overrepresented Biological Processes, Molecular Functions, Pathways, and Upstream Regulators of Differentially Abundant Transcripts in Male Compared to Female Conceptuses on Day 19

In total, sex of the conceptus significantly affected 102 biological processes more than would be expected by chance with 68 molecular functions associated with conceptus sex on Day 19. A full list of the overrepresented biological processes and molecular functions and their associated genes are given in Supplemental Tables S3 and S4. IPA identified 407 pathways that were overrepresented in this list of differentially abundant transcripts, the details of which are available in Supplemental Table S5. Included in these pathways were those associated with cell cycle progression (i.e., role of CHK proteins in cell cycle checkpoint control, 32 DEGs; cell cycle control of chromosomal replication, 14 DEGs, cell cycle: G2/M DNA damage checkpoint regulation, 22 DEGs), embryonic cell lineage (i.e., mouse embryonic stem cell pluripotency, 36 DEGs; role of NANOG in mammalian embryonic stem cell pluripotency, 40 DEGs; telomerase signaling, 35 DEGs; human embryonic stem cell pluripotency, 32 DEGs, DNA methylation and transcriptional repression signaling, eight DEGs) as well as pathways involved in conceptus-maternal interactions (i.e., role of PKR in interferon induction and antiviral response, 18 DEGs; GM-CSF signaling, 24 DEGs; androgen signaling, 39 DEGs; glucocorticoid receptor signaling, 81 DEGs; HGF signaling, 37 DEGs; MIF regulation of innate immunity, 11 DEGs; MIF-mediated glucocorticoid regulation, eight DEGs).

Interestingly, 23 of these pathways were associated with amino acid degradation, biosynthesis, and signaling. In total, 66 DEGs were involved in the mTOR signaling pathway, six were involved in tryptophan degradation X, five involved in methionine degradation I, five in the pathway of phenylalanine degradation IV, with seven, five, and two DEGs associated with the pathways of isoleucine degradation I, leucine degradation I, and lysine degradation II, respectively.

Analysis of upstream regulators of the DEGs in male compared to female conceptuses revealed that 55 of these DEGs were identified as significant upstream regulators of other DEGs due to the sex of the conceptus (Supplemental Table S6). Moreover, a significant proportion of these were transcriptional regulators (16 in total). Additional molecules identified as upstream regulators of genes that were different by virtue of conceptus sex included the amino acids serine and glutamine as well as interferon alpha.

Differences in Amino Acid Composition of ULF Recovered from Uteri with Male or Female Conceptuses

To assess whether conceptus sex affected the amino acid composition of ULF, we examined the amino acid content of ULF recovered from heifers with either a male or a female conceptus on Day 19. Of the 18 amino acids analyzed in the ULF-containing female or male conceptuses, the concentrations of arginine, asparagine, glutamine, histidine, isoleucine, lysine, methionine, and tryptophan were significantly higher ($P < 0.05$: Fig. 2) in the ULF containing a male compared to a female conceptus on Day 19.

Quantitative RT-PCR Validation of Microarray Data and Expression of Amino Acid Transporters in the Endometrium and Conceptus on Day 19

Consistent with the microarray analysis, there was no difference in the abundance of any of 26 amino acid transporters analyzed between endometria from cattle gestating a male compared to a female conceptus ($P > 0.05$). Expression of *XIST* was higher ($P < 0.001$) in the endometrium of all

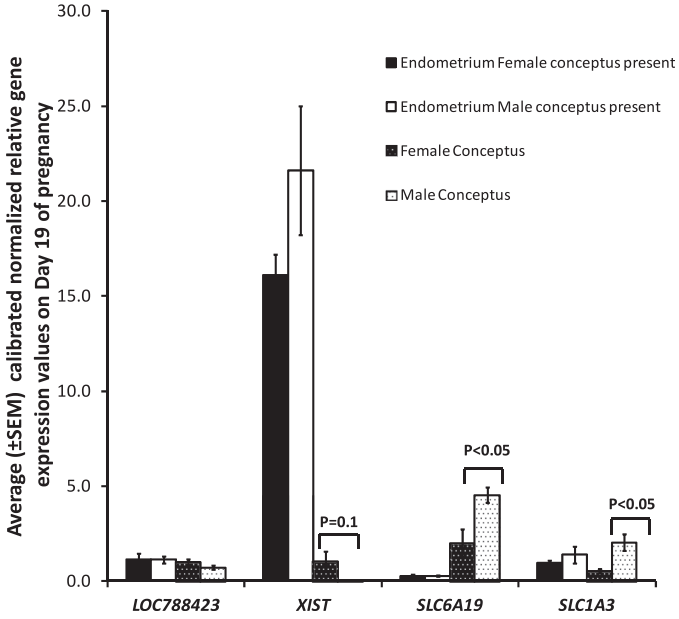

FIG. 3. Analysis of transcript expression for amino acid transporters and selected transcripts from the microarray study qRT-PCR in male and female conceptuses on Day 19 of pregnancy. All expression values are given as mean calibrated, normalized, relative expression values in arbitrary units (AU ± SEM) for n = 5 male (closed bars) and n = 5 female (open bars) conceptuses on Day 19. Significant differences in expression between male and female conceptuses are noted by an asterisk (*) when $P < 0.05$.

animals analyzed compared to conceptus expression but did not differ between groups ($P > 0.05$).

Comparison of transcript abundance between male and female conceptuses revealed a higher expression of *XIST* in female compared to male conceptuses ($P = 0.01$) with a large range in expression values in female conceptuses (range 0.118–2.62 CNRQ) compared to males (range: 0.004–0.016 CNRQ) while abundance in the endometrial tissue was substantially higher (range: 12.35–33.37 CNRQ). The expression of the amino acid transporters *SLC6A19* and *SLC1A3* was significantly higher in male compared to female conceptuses on Day 19 (Fig. 3).

Comparison of Sexually Dimorphic Transcript Abundance in Day 19 Conceptuses with That in the Day 7 Blastocyst

In order to identify the temporal changes in sexually dimorphic gene expression that occur between the blastocyst stage (Day 7) and implantation (Day 19), the same preprocessing and stringency measures were applied to previously published data from our group [13]. This resulted in the identification of 2295 DEGs between male and female Day 7 blastocysts, 1176 of which were higher in the male, and 1119 were higher in female blastocysts. Of these, 7.1% of the DEGs (163) in the blastocyst linked with sex were located on the X chromosome in comparison to the 2.7% of X-chromosome-associated genes on Day 19 of conceptus development (Fig. 4). A similar number of DEGs were identified on each chromosome as a proportion of the total number of genes on each chromosome. A comparison of these DEGs revealed 1392 were only differentially abundant on Day 7 (659 increased in male and 904 increased in female embryos) while 4239 genes were altered on Day 19 (2418 increased in male and 2210 increased in female conceptuses). Only 862 genes were differentially abundant in the embryo/conceptus on both Days 7 and 19 (Fig. 5A). These transcripts separated into four main categories: 164 DEGs were affected in the same way, that is, increased in male embryo compared to the female embryo on both Days 7 and 19, while 203 were increased in the female

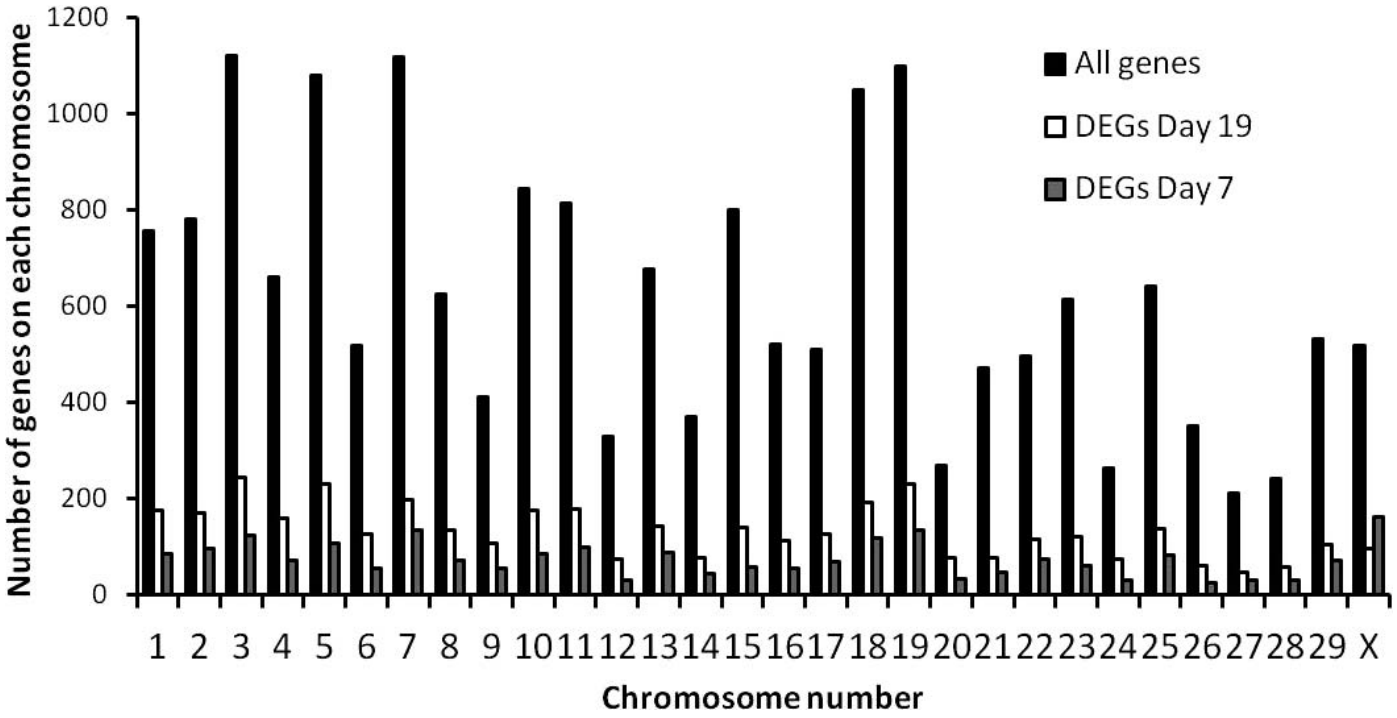

FIG. 4. Graph depicting the number of genes on each chromosome as well as the frequency of chromosomal location of genes identified as differentially expressed between male and female conceptuses on Day 19 of pregnancy (this study) and embryos on Day 7 of pregnancy [13].

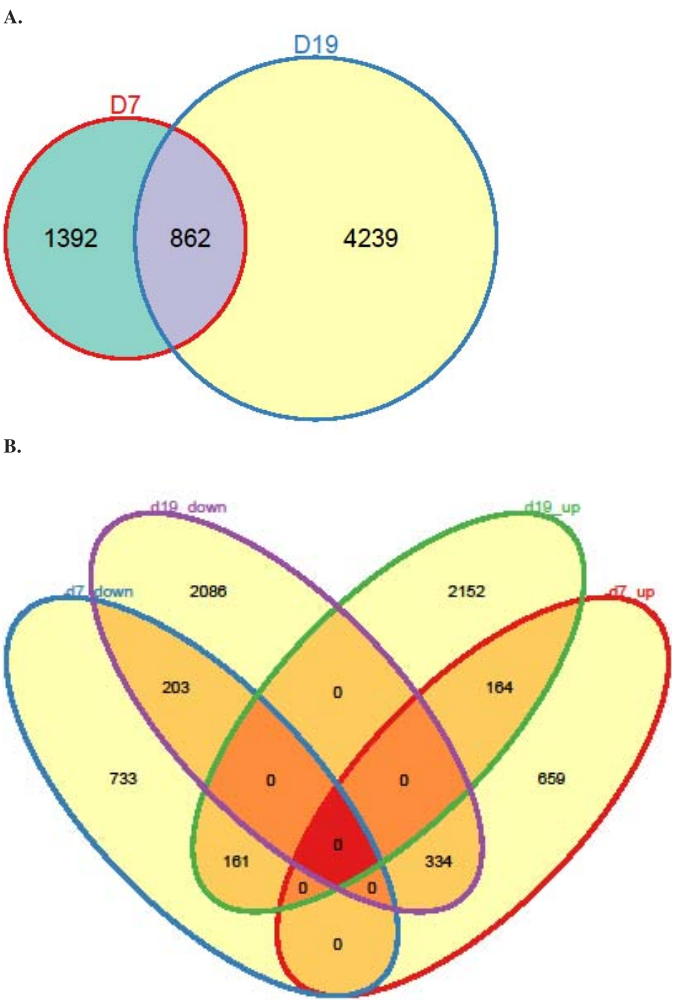

FIG. 5. Venn diagram showing the overlap in differentially expressed genes (DEGs) (**A**) as well as the direction of fold change between male and female embryos (**B**) using the same analysis criteria (Day 7 [13]) and male and female conceptuses (Day 19: this study). All data was subjected to the same stringent data preprocessing prior to generation and comparison of lists of DEGs.

embryo/conceptus on both days. In contrast, 161 transcripts were increased in female embryos on Day 7 and decreased on Day 19 compared to male embryos, while for 334 genes the expression pattern was the opposite, that is, decreased in female embryos on Day 7 and increased on Day 19 compared to male embryos (Fig. 5B; full information on fold change and *P*-values are given in Supplemental Table S7). The location of these temporally altered transcripts was notable because 21.2% of the DEGs that were increased in female compared to male embryos on Days 7 and 19 and 14.9% of the DEGs that were increased in female embryos on Day 7 and decreased in female embryos on Day 19 were located on the X chromosome (Supplemental Table S8).

Endometrial Transcriptomic Response to a Male Versus Female Conceptus

Despite major differences in Day 19 conceptus gene expression, no differences in the endometrial transcriptomic response to a male or female conceptus were observed ($P > 0.05$; data accessible in Gene Expression Omnibus database GSE75754). Consistent with this finding, the amount of IFNT present in the uterine flush from which a male (335 687 ± 68,118 AU) or female (279 413 ± 55,797 AU) conceptus was recovered was not different ($P > 0.05$).

DISCUSSION

This study builds on the knowledge that one-third of transcripts present at the blastocyst stage of development in cattle are regulated by the sex of the embryo [13] as well as the novel hypothesis that the endometrium is a biosensor of the developmental competency or origin of the embryo [24] by addressing whether this sexual dimorphism is maintained throughout the elongation period of conceptus development and/or whether the endometrium is sensitive to the sex of the developing conceptus on Day 19. Using large-scale transcriptomic analysis, we have determined that more transcripts are differentially abundant between male and female conceptuses on Day 19 than in blastocysts on Day 7, but some differences (approximately 7%) are maintained between these two distinct morphological stages of embryo/conceptus development. Proportionally, more transcripts on autosomal chromosomes are modulated by sex as opposed to just the X chromosome on Day 19 compared to Day 7. Despite these differences, the endometrium does not respond differently to the presence of a male or a female conceptus, at least not at the transcriptome level after pregnancy recognition has occurred, and production of IFNT is not different. However, sex of the conceptus does affect the availability of amino acids in the ULF, likely due to the different requirements of the conceptuses for amino acids in a sex-dependent manner.

The significant number of DEGs between male and female conceptuses was a surprising finding for a number of reasons. First, a number of studies in the literature have found little difference in transcript abundance between male and female conceptuses. While the lack of a significant effect of sex on gene expression is somewhat at odds with the current study, the design of these studies was very different and the platforms used were not always comparable. The study design in the paper of Degrelle et al. [25] was radically different from the present study. They compared Day 18 somatic cell nuclear transfer (SCNT)-derived conceptuses (n = 30) to those derived by artificial insemination (AI) (n = 10) or in vitro production (IVP) (n = 10). All of the SCNT conceptuses were female, one-half of which showed signs of atypical elongation and gastrulation. To generate SCNT and IVP conceptuses, five to six blastocysts were transferred per recipient. For the AI controls, animals underwent a mild superovulation protocol (600 IU PMSG), and they also used a different microarray platform. The absence of a sex effect was based on comparison of conceptuses derived from AI (males versus females) as well as females only (AI, IVP, and SCNT). Similarly, Betscha et al. [26] compared gene expression in Day 16 conceptuses derived by AI, SCNT, or IVP bovine using the Affymetrix bovine genome array. In this case, all SCNT embryos were male. While the authors failed to detect differential gene expression amongst the AI and IVP embryos, this was based on analysis of only one female and two male embryos in each case. Valour et al. [27] investigated the effect of dam physiological status on embryo development and conceptus gene expression in growing heifers and postpartum cows using a homemade 22K bovine oligonucleotide probe array. Sex of the conceptus explained 3.1% of the variability observed in the overall gene expression pattern. Conversely, the physiological status of the dams represented 23.7% of the variability. However, in these studies, both the experimental design and platform differed from this study.

Second, the X chromosome that is silenced is always the paternal X chromosome in extraembryonic membranes, that is, in the EET (reviewed by [28]). There are substantial data in the literature demonstrating a sex-dependent rate of growth/development. However, these data refer to development to the blastocyst stage only, and to our knowledge cell number in elongated conceptuses has not been quantified. In as much as was possible to assess, conceptus length was not different between male and female conceptuses in this study. This is difficult to measure accurately at Day 19 because of the fragile nature of the conceptus at this stage and the fact that the conceptuses are often tangled due to the flushing recovery technique. However, two points would suggest that male conceptuses were not advanced compared to females. First, IFNT content of ULF, which is highly correlated with conceptus length and trophectoderm cell number [29], was not different between males and females. Second, the expression of a number of marker genes of gastrulation in the EET cells, as detailed in the study by Degrelle et al. [25], specifically the genes *CPA3, CALM1,* and *HNRNPDL,* were not differentially expressed between male and female conceptuses in this study (Supplemental Table S2). Despite the fact that the proportion of DEGs located on the X chromosome was lower than those previously reported in the bovine blastocyst [13], similar numbers of DEGs were located on the X chromosome. Sexual dimorphic biological pathways in Day 19 conceptuses included cell cycle progression and chromosomal replication as well as stem cell pluripotency. Also overrepresented are genes involved in the pathways of glucocorticoid receptor signaling, androgen signaling, and MIF signaling. These data are interesting in the context of a recent study by Dobbs et al. [30] that found a sex-dependent response of the embryo to colony-stimulating factor 2. Thus, embryo trophic factors produced by male and female conceptuses on Day 19 may affect pathways in the conceptus itself in a sex-specific manner.

Comparisons of transcripts affected by sex of the embryo at Day 7 to those affected in the Day 19 conceptus revealed that only a small proportion are conserved between these distinct morphological time points (Fig. 4). The timing and mechanism of XCI differs between germ line and somatic cells [12, 28], and this may account for the seemingly small overlap in sex-regulated genes. In particular, tissue analyzed on Day 7 consists of a mixture of approximately 2:1 trophoblast to inner cell mass, whereas on Day 19 only trophoblast cells were analyzed. Within these 864 genes, less than half displayed a similar expression pattern on both days of pregnancy. Interestingly, a number of genes were decreased on both Days 7 and 19 in the female compared to male embryo/conceptus, that is, when XCI has occurred, given increased expression of *XIST* in the female embryo [14] and conceptus (this study). Evidence from other species shows that coordinate with XCI, portions of autosomal chromosomes can become inactivated during this process (reviewed by [31]). The decreased transcript abundance in female conceptuses on Day 19 may be autosomal genes inactivated during the process of XCI. In addition, it is possible that the transcripts only decreased on Day 19 in female conceptuses may be a late or delayed consequence of XCI. Some caution is needed with the interpretation of these comparisons, however, given the two data sets are derived from somewhat different sources. The Day 7 blastocyst data were derived from in vitro fertilized embryos produced with sex-sorted semen. The current data were derived from AI with conventional (non-sex-sorted) semen.

In contrast to the sex-induced differences in the conceptus, no differences in the endometrial transcriptomic response to either a male or female conceptus were detectable on Day 19. As has been shown on Day 18 [11] and Day 20 [10] in cattle and in humans [32], the endometrium does respond differently to conceptuses of differing quality and trajectories with regard to pregnancy outcome [10, 11]. Therefore, the lack of differences observed in the endometrial transcriptomic response to male and female conceptuses may simply reflect a lack of difference in developmental competency of a male versus a female conceptus after Day 19. It is interesting, however, that a number of the DEGs between male and female conceptuses are also expressed in the endometrium (e.g., *MIF, OXT, SI1*). In addition, we know from previously reported studies that the endometrium expresses receptors for some conceptus-derived ligands, but the fact remains that no differences in the pregnancy recognition signal occur, at least in the intercaruncular endometrium. However, it is possible that there may be protein differences, or differences in the posttranslational modifications of proteins in the endometrium in response to male and female conceptuses, and this could be an avenue of future study.

Both male and female Day 8 blastocysts [33] as well as Day 14 conceptuses [14] display differences in the type of IFNT transcripts expressed; however, there was no difference in the abundance of IFNT in the ULF containing male or female conceptuses on Day 19. Given that IFNT is predominantly responsible for the pregnancy-recognition response in the endometrium of cattle, because there are no differences between male and female abundance of IFNT, this may explain in part why there is no difference in the transcriptomic response of the endometrium to the conceptus. Differences in amino acid composition in the ULF on Day 19 were interesting. In a previous study by Sturmey et al. [16], male in vivo-derived blastocysts had a lower depletion of amino acids and lower amino acid turnover compared to female blastocysts, that is, increased amounts of amino acids in the media similar to increased amino acids in the ULF in this study. If increased amino acids in the ULF are indicative of reduced uptake by the Day 19 conceptus, which is likely given there are no differences in amino acid transporters in the endometrium, then it is reasonable that male conceptuses do not utilize amino acids to the same extent as female conceptuses. Differences in the amino acid composition of the ULF hosting cloned and IVP conceptuses on Day 18 have been observed [34], but this is in line with reduced expression of amino acid transporters in the endometrium (likely due to the different endometrial response these conceptuses elicit [11]). In the present study, differences in amino acid composition were not due to differences in transcript abundance for the amino acid transporters in the endometrium. Thus, despite a similar uterine environment, male and female conceptuses utilize this environment in a sex-specific manner in vivo similar to the phenomenon observed in vitro with regard to amino acid uptake [16] as well as other energy substrates. Indeed, concentrations of the neutral amino acids asparagine, glutamine, and methionine were higher in the ULF hosting male compared to female conceptuses. Male conceptuses exhibited increased expression of the neutral amino acid transporter *SLC6A19*. This increased expression of the transporter in the male conceptuses may not necessarily translate into increased neutral amino acid uptake. Alternatively, this could be increased transcript abundance that may translate into increased amino acid uptake by the male conceptus at a later time point, that is, after Day 19 when these samples were taken.

In conclusion, the results of this study support the hypothesis that XCI is incomplete during the initiation of implantation in cattle. There is also significant sexual dimorphism in terms of amino acid consumption as well as gene expression in the conceptus at Day 19. Yet despite the

significant difference in gene expression changes, the sex of the conceptus itself does not elicit a significantly different response in the transcriptome of the endometrium, at least on Day 19. Moreover, given the fact the conceptus is exposed to the same maternal environment, because there is no difference in endometrial response, we propose that conceptuses of different sexes utilize the same uterine environment but in a sex-dependent fashion.

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