Research Article

Interferon tau-dependent and independent effects of the bovine conceptus on the endometrial transcriptome[†]

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Abstract

This study investigated bovine conceptus-induced modifications to the endometrial transcriptome related to effects of interferon tau (IFNT), conceptus origin (in vivo vs. in vitro), and conceptus sex. In vitro (IVF) or in vivo (superovulation and artificial insemination, AI) produced blastocysts were transferred into recipient heifers on day 7 of the estrous cycle. On day 15, IVF- or AI-derived conceptuses were obtained by uterine flushing and individually placed on endometrial explants in media for 6 h. Explants were also cultured with media alone as a control or media containing 100 ng/mL IFNT. Total explant RNA was analyzed by RNA-Seq. Incubation of endometrium with IFNT or IVF- or AI-derived conceptuses changed ($P \leq 0.001$) expression of 491, 498, and 576 transcripts, respectively, compared to the control. Further, 369 differentially expressed genes (DEGs) were common between explants exposed to IFNT or a conceptus. A total of 240 DEGs were uniquely altered by conceptuses (IVF- and AI-derived) but not IFNT. Of these transcripts, 46 were shared between the IVF and AI groups, while 61 and 133 were specific to IVF and AI conceptuses, respectively. Five genes [melanophilin (*MLPH*), prominin-2 (*PROM2*), myeloid associated differentiation marker (*MYADM*), vomeronasal 1 receptor 4 like (*VN1R4L*) and 5-hydroxytryptamine receptor 1A (*HTR1A*)] were more abundant in endometrium exposed to female compared to male conceptuses ($P < 0.001$). A single gene [ADP-ribosylation factor like GTPase 4C (*ARL4C*)] was more abundant in response to male conceptuses ($P < 0.001$) than female conceptuses. These data support the hypothesis that conceptus regulation of gene expression in the endometrium is complex and involves factors other than IFNT that may have a biological role in pregnancy establishment.

Summary Sentence

Elongating bovine conceptuses modify the adjacent endometrial transcriptome in a manner dependent and independent of interferon tau and related to conceptus origin and sex.

Key words: endometrium, transcriptome, conceptus, interferon tau, explant, RNAseq.

Introduction

Optimal dialog between the developing embryo and mother during the peri-implantation period is essential for pregnancy recognition and uterine receptivity, ultimately setting the stage for implantation and placentation [1, 2]. In contrast to primates and rodents, in which implantation occurs shortly after the blastocyst enters the uterus [3], in ungulates, such as ruminants and pigs, the early conceptus undergoes a phase of rapid growth and elongation before implantation, the latter occurring 2–3 weeks after fertilization [4, 5]. In cattle, conceptus elongation is initiated around day 13 of gestation when the conceptus sequentially transitions from a spherical into an ovoid, tubular, and then filamentous form [1, 6]. Elongation is necessary to ensure sufficient concentrations of interferon tau (IFNT), the maternal recognition of pregnancy signal, are secreted and to expand the conceptus surface area for maximal vascular exchange with maternal tissues after implantation [7, 8]. An inability of the conceptus to optimally elongate undoubtedly results in embryonic loss and is believed to significantly contribute to reproductive failure in cattle [9–11].

It is generally accepted that blastocysts produced in vitro are inferior in quality to in vivo-derived embryos. This is evident in terms of ultrastructure [12], gene expression profiles [13–15], cryotolerance [16], and pregnancy rate after transfer [17]. This difference is reflected in the fact that in commercial embryo transfer, the majority of in vitro-produced blastocysts are transferred fresh while the majority of in vivo-derived blastocysts are transferred frozen [18]. Whether the interaction between the elongating conceptus and the maternal endometrium is compromised with in vitro-produced embryos is unknown. The transcriptome of the bovine endometrium has been described under a variety of physiological and experimental conditions [11, 19–21]. Temporal changes in uterine gene expression occur irrespective of whether the cow is pregnant or not and it is only during maternal recognition of pregnancy, around day 16, when major changes in gene expression between cyclic and pregnant endometrium become apparent [20, 22]. Both ovarian progesterone (P4) and conceptus IFNT greatly influence the endometrial transcriptome. Progesterone is essential to regulate reproductive cyclicity and uterine receptivity, and maintain pregnancy [23]. In cattle, sufficient concentrations of IFNT must be released by the elongating conceptus between days 15 and 17 to block pulsatile secretion of endometrial prostaglandin F₂ alpha (PGF₂α), the luteolytic factor responsible for regression of the corpus luteum (CL) and associated decline in P4 [10, 24]. As a result, endometrial responses to IFNT, particularly expression of IFN-stimulated genes (ISG) such as interferon-stimulated gene 15 (ISG15), myxovirus resistance 1 (MX1), myxovirus resistance 2 (MX2), and 2'-5'-oligoadenylate synthase 1 (OAS1) can be detected in endometrium from day 15 and day 16 pregnant cattle [20, 25].

Recent proteomic studies of day 16 bovine conceptus secretory proteins have identified factors in addition to IFNT that are released by early elongating conceptuses and that could modify the conceptus-endometrial microenvironment [26]. However, studies identifying endometrial responses to these factors or to the early elongating conceptus, independent of IFNT, are limited and/or challenging to execute [20, 25]. Ex vivo elongation of a conceptus has not been achieved and studies designed to investigate conceptus elongation require extensive in vivo experiments. Furthermore, collection of endometrial tissue once adjacent to an early elongating conceptus

is a challenge using conventional uterine flushing and endometrial collection practices.

It has been elegantly shown that the endometrium can act as a “sensor,” with its transcriptome reflective of the type and developmental competency of the conceptus present [27, 28]. Comparison of endometrial responses to bovine conceptuses produced by somatic cell nuclear transfer, in vitro fertilization (IVF), or artificial insemination (AI) suggest that placental failure in bovine clone pregnancies may originate from abnormal embryo–maternal communication during the peri-implantation period (day 18–20) [27, 28]. Similar data indicating the ability of the endometrium to act as a “sensor” of embryo developmental competency have been reported in humans [29].

Recently, endometrial explants were used to model innate immune responses during the estrous cycle in cattle [30]. Endometrial explants may also be used to study local conceptus–maternal dialog, as they permit the examination of tissue that has been in direct contact with the conceptus. We recently reported increased expression of ISG in bovine endometrial explants exposed to bovine blastocysts (Passaro et al. [31]). Here, we utilized a conceptus–endometrial explant co-culture system to study the dialog between elongating conceptuses and the endometrium. We hypothesized that the endometrium responds differently to conceptuses of different origins. Specifically, the objective was to compare the transcriptomic response of the endometrium following exposure to IFNT or a conceptus derived from the transfer of an in vivo-derived or in vitro-produced blastocyst in order to identify novel transcripts dependent and independent on IFNT, conceptus origin, and conceptus sex. Such studies investigating elongation of the early bovine conceptus and its endometrial environment will help delineate biological pathways that contribute to pregnancy success or, alternatively, reproductive failure, in cattle.

Materials and methods

All experimental procedures involving animals were sanctioned by the Animal Research Ethics Committee of University College Dublin and were licensed by the Health Products Regulatory Authority, Ireland, in accordance with Statutory Instrument No. 543 of 2012 (under Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes).

Experimental design

The experimental design is illustrated in Figure 1. In vivo and in vitro-produced bovine blastocysts were transferred in groups of 10 to synchronized recipient heifers on day 7. Elongated conceptuses were recovered on day 15 and co-cultured with endometrial explants to detect conceptus-induced changes in gene expression. Explants were cultured for 6 h with (i) medium alone (control; n = 6 explants); (ii) medium containing 100 ng/mL of recombinant ovine IFNT (n = 6 explants); (iii) a day 15 conceptus derived from the transfer of in vivo-derived blastocysts (AI, n = 4 explants); or (iv) a day 15 conceptus derived from the transfer of in vitro-produced blastocysts (IVF, n = 7 explants).

In vivo production of bovine blastocysts

Estrous cycles of crossbred beef heifers (predominantly Charolais and Limousin cross, n = 25) were synchronized using an 8-day

Figure 1. Experimental design. In vivo -derived (A) and in vitro-produced (B) bovine blastocysts were transferred in groups of 10 to synchronized recipient heifers on day 7 and elongated conceptuses were recovered on day 15 (B) and co-cultured with endometrial explants (C) to detect conceptus-induced changes in gene expression. P4 = progesterone releasing device; GnRH = gonadotrophin releasing hormone; PG = prostaglandin F2 α ; FSH = follicle-stimulating hormone; AI = artificial insemination; ER = embryo recovery; ET = embryo transfer; IVM = in vitro maturation; IVF = in vitro fertilization; IVC = in vitro culture; D7 Blast = day 7 blastocyst; CR = conceptus recovery; Exp 6 h = in vivo and in vitro conceptuses cultured on top of an endometrial explant for 6 h. Day 0 was considered the day of fertilization (28–32 h after the start estrus behavior).

P4 releasing intravaginal device (PRID®E, 1.55 g P4; Ceva Santé Animale). On the day of PRID insertion, heifers received a 2 mL intramuscular injection (i.m.) of a gonadotropin releasing hormone analog (GnRH; Ovarelin®, Ceva Santé Animale, equivalent to 100 μ g Gonadorelin) and one day prior to PRID removal they received a 5 mL i.m. injection of prostaglandin F2 α (PGF2 α ; Enzaprost®, Ceva Santé Animale). Ten days after standing estrus, heifers were superovulated by twice daily i.m. injections of follicle-stimulating hormone (FSH; Folltropin®, Bioniche Animal Health) for 4 days in conjunction with an injection of PGF2 α on the third day of FSH followed by AI with frozen-thawed semen from a bull of proven fertility. Heifers were slaughtered at a local abattoir 7 days later, and each uterine horn was gently flushed with 20 mL of pre-warmed PBS (38.8°C) containing 5% fetal calf serum (FCS) to collect in vivo-produced blastocysts. For both donors and recipients (below), day 0 was considered the day of fertilization, estimated to be approximately 28–32 h after estrus onset [32–34].

In vitro production of bovine blastocysts

In vitro-produced blastocysts were obtained as previously described by Rizos et al. [16]. Briefly, ovaries were collected from a local abattoir and surface visible follicles were aspirated with an 18-gauge needle and syringe. Cumulus–oocyte complexes (COC) were matured, in groups of 50, in TCM-199 media supplemented with 10%

FCS and 10 ng/mL epidermal growth factor at 38.8°C in 5% CO₂ in air for 24 h before fertilization. Frozen-thawed semen from the same bull as used for the generation of in vivo-derived blastocysts (described above) was centrifuged through a Percoll gradient and washed before matured COCs were inseminated at a sperm concentration of 1×10^6 spermatozoa/mL of fertilization medium (day 0). Gametes were co-cultured for approximately 24 h at 38.8°C in 5% CO₂ and air. The next day (day 1 of development), presumptive zygotes were denuded and cultured in synthetic oviduct fluid medium containing 5% FCS at 38.8°C in 5% O₂ and 5% CO₂ for 6 days.

Generation of day 15 conceptuses

To produce day 15 conceptuses, in vivo and in vitro-derived blastocysts were transferred in groups of 10 to the uterine horn ipsilateral to the CL of crossbred recipient heifers (n = 31) which had been synchronized as described above (Figure 1). Recipients were slaughtered in a commercial abattoir on day 15 of the estrous cycle, and their reproductive tracts were flushed (as described above) to collect conceptuses. The number and dimensions (length and width) of recovered conceptuses were recorded.

Endometrial explant culture

Reproductive tracts (n = 7) in the late luteal phase of the estrous cycle, based on the classification described by Ireland et al. [35],

Figure 2. A representative image of an elongated conceptus cultured on top of an endometrial explant. Conceptuses and endometrial explants were co-cultured in 1 mL of Roswell Park Memorial Institute (RPMI) media for 6 h before tissues were collected and snap-frozen for RNA (explant) and DNA (conceptus) extraction.

were collected from a local abattoir and placed on ice before transport to the laboratory (within approximately 1 h of slaughter). Intercaruncular endometrial explants were prepared as described by Borges et al. [30]. Briefly, once in the laboratory, the uterine horn ipsilateral to the CL was dissected away from the rest of the tract and opened longitudinally on the anti-mesometrial side to expose the endometrium. The endometrium was washed with 1% PBS (1% ABAM; Gibco, ThermoFisher Scientific) before an 8 mm biopsy punch was used to dissect completely through the intercaruncular uterine tissue from the middle third of the uterine horn. Sterile scissors were then used to dissect the endometrium away from the myometrium. Once dissected, the explants (50–80 mg) were washed in Hank balanced salt solution (HBSS; Gibco, ThermoFisher Scientific) containing 1% ABAM before placement in culture wells (4-well plate) containing 1 mL of Roswell Park Memorial Institute (RPMI) medium (Gibco, ThermoFisher Scientific) with 1% ABAM. Explants were cultured (one per well) endometrial side up in 5% CO₂ and air at 38.8°C for 1 to 5 h before use. Before conceptuses were added to the explants, the medium was completely replaced with fresh medium (38.8°C) (Figure 2). In order to minimize variation, explants from the same uterus were used across all treatments in a given replicate. Information regarding conceptus length and sex can be found in Supplemental Table S1. After the 6 h of incubation, conceptuses were removed from the explant and snap-frozen in liquid nitrogen. Explants were snap-frozen in liquid nitrogen and stored at –80°C for RNA extraction. The 6-h culture duration was chosen based on a previous study from our group in which we cultured day 8 blastocysts ($n = 20$) with explants for 6 h culture vs 24 h culture and did not detect any differences in response [31]. Furthermore, this relatively short co-culture avoids compromising the integrity of the tissue, as variable tissue degradation has been reported after prolonged culture of explants [30].

Conceptus DNA extraction and sexing

Conceptus DNA was extracted and concentration assessed using the DNeasy blood and tissue kit (Qiagen) and Nanodrop 1000 (ThermoFisher Scientific), respectively, following the manufacturer's recommendations. Approximately 50 ng of DNA

and a single primer pair were used during PCR to amplify a segment of exon 5 within the bovine Amelogenin gene located on the sex chromosomes [36]. The forward and reverse primers used were 5'-GGCCAACACTCCATGACTCCA-3' and 5'-TGGGGAATATTGGAGGCAGAG-3', respectively. The 20 μ L PCR reactions (power SYBR green; Roche Diagnostics Ltd) consisted of an initial temperature of 95°C for 10 min followed by 40 cycles consisting of melting at 95°C for 15 s and annealing and extension at 60°C for 1 min using the 7300 Real Time PCR System thermocycler (Applied Biosystems, ThermoFisher Scientific). The PCR amplicons and DNA ladder were electrophoresed through a 2% agarose gel in 0.09 M Tris-borate and 0.002 M ethylenediamine tetra-acetic acid buffer containing 0.5 μ g/mL ethidium bromide at 100 V for approximately 1.5 h before visualized with UV light. As previously described by Gokulakrishnan et al. [36], a 64 bp deletion within the Amelogenin gene on the Y chromosome results in amplification of a smaller DNA product (178 bp) during PCR of male conceptus DNA, allowing separation of X (241 bp) and Y chromosome amplicons during gel electrophoresis.

Explant RNA extraction, RNA-sequencing, and RT-qPCR

Total RNA extraction was carried out as described by Forde et al. [37]. Briefly, total RNA was extracted from approximately 50 mg of endometrial explant tissue using Trizol reagent (Invitrogen) as per the manufacturer's instructions. On-column RNA clean-up was performed using the Qiagen mini kit (Qiagen). The quantity and quality of RNA were determined using the Nano Drop 1000 (Thermo Fisher Scientific) and the Agilent Bioanalyzer (Agilent Technologies), respectively. The RNA integrity number $\pm$ the standard deviation was 7.4 ± 0.3 . RNA concentration was determined by quantitative high-sensitivity RNA analysis on the Fragment Analyzer instrument (DNF-472; Advanced Analytical Technologies, Inc.). RNA library preparation and sequencing was conducted by the University of Missouri DNA Core facility as described by Moraes et al. [11].

Raw sequences (fastq) were subjected to quality control using windowed adaptive quality trimming approach implemented in fqtrim (<https://ccb.jhu.edu/software/fqtrim/>). The quality reads were then mapped to the bovine reference genome UMD3.1 using Hisat2 mapper (<https://ccb.jhu.edu/software/hisat2/>), which is a fast and sensitive alignment program for next-generation sequencing data [38]. Read counts mapping to each gene were determined from the mapping data using FeatureCounts [39]. Differential expression analysis between sample groups was performed by robustly fitting the expression data to a generalized linear model using edgeR [40].

The DAVID Bioinformatics Resource 6.8 was used to carry out a Gene Ontology (GO) analysis of transcripts found to be statistically significant during the RNA-Seq analysis [41]. An enriched biological process (BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were included. The GO direct category was used during the annotation and provides mappings directly annotated by the source database. The *Homo sapiens* background was used during the analysis, and GO direct and KEGG categories were considered enriched when $P \leq 0.05$.

In preparation for RT-qPCR, 500 ng of explant mRNA was reverse transcribed into cDNA using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, ThermoFisher Scientific) in a 20 μ L reaction following the manufacturer's recommendation. The RT-qPCR reactions (20 μ L; 93.75 ng RNA equivalent) were carried out using the Roche SYBR Green RT-qPCR master mix kit (Roche Diagnostics Ltd) and the Applied Biosystems 7500 Fast Real-Time

PCR system (ThermoFisher Scientific) following the manufacturer's recommendations. The PCR primer amplification efficiencies (E) for each PCR target sequence were calculated from the standard curve generated from seven cDNA dilutions (Supplemental Table S2). The equation used for the efficiency calculation was $E = [10^{(-1/\text{slope})} - 1]$. Percent efficiency was calculated by dividing E by 2 and then multiplying by 100. A dissociation analysis was included for each primer pair to evaluate primer specificity for the target sequence.

The qbase + computer program (Biogazelle, Zwijnaarde, Belgium) was used to calculate the normalized relative expression quantities (relative expression) of target genes based on a generalized delta-delta quantification cycle method ($\Delta\Delta C_q$; also known as $\Delta\Delta C_T$) [42]. To identify potential normalization targets, RT-qPCR was carried out for eight potential genes across a subset of samples representing all treatments. Genes peptidylprolyl isomerase A (*PPIA*) and succinate dehydrogenase complex flavoprotein subunit A (*SDHA*) were found to be the most stably expressed across treatments using the geNorm analysis (geNorm M < 0.25; Supplementary Table S2) [43]. The RT-qPCR thermo cycler settings for all reactions consisted of an initial temperature of 95°C for 10 min, followed by 40 PCR cycles consisting of melting at 95°C for 15 s and annealing and extension at 60°C for 1 min.

A general linear model procedure (proc GLM) in the statistical analysis software (SAS) was used to analyze the explant, RT-qPCR, and relative expression data. Two statistical analyses were included: (1) testing the effect of IFNT and conceptus origin (AI or IVF), and (2) testing the effect of conceptus sex on endometrial gene expression. Data residuals were scrutinized for normality using the PLOTS = (diagnostics residuals) statement. If the data residuals were not normally distributed, they were corrected using a log base-10 (log10) or square root (sqrt) transformation. All relative expression values are presented in their original format; however, in the case of a transformation, the statistical analysis is of the transformed data. Four treatments were included in the first analysis including control, IFNT, AI, and IVF-derived conceptuses. A Tukey-Kramer adjustment was included to account for multiple comparisons. Two orthogonal contrast statements were included. The orthogonal contrasts were: Contrast 1 (C1), media only exposed endometrium (control) vs. conceptus (AI and IVF)-exposed endometrium and Contrast 2 (C2), IFNT-exposed endometrium vs. conceptus (AI and IVF)-exposed endometrium. Data are presented as least squares means $\pm$ standard error of the least squares mean (LSM $\pm$ SEM). Statistical significance was declared at a $P \leq 0.05$.

Results

Differentially expressed genes

Analysis of the RNA-Seq data resulted in identification of 491, 498, and 576 differentially expressed transcripts (up- or downregulated) in endometrium exposed to IFNT, IVF-, or AI-derived conceptuses, respectively, compared to control endometrium ($P \leq 0.001$; Supplementary Excel File 1). In an attempt to identify a gene of origin for unidentified transcripts (89, 85, and 106 for IFNT, IVF, and AI groups, respectively), the unidentified transcript sequences were aligned (BLAST) with sequences published in Ensembl and National Center for Biotechnology Information (NCBI) databases. Some of the uncharacterized transcripts corresponded to a single gene (>98% homology), resulting in identification of 466, 479, and 548 differentially expressed genes (DEGs) in co-cultured endometrium (IFNT, IVF, or AI-derived conceptus, respectively). The

Figure 3. A venn diagram (Venny 2.1.0 BioinfoGP) identifying differentially expressed genes (DEGs) in endometrium treated with ovine interferon tau (IFNT), in vitro fertilization (IVF), and artificially inseminated (AI)-derived conceptuses compared to control endometrium. The venn diagram indicates genes differentially expressed in response to all three treatments and related to the maternal recognition of pregnancy signal, IFNT. The large box below the venn diagram lists the ensemble gene ID, gene name, and log fold change (LogFC) in expression (compared to control endometrium) for the 10 most upregulated (greatest to least) and 10 most downregulated (from least downregulated to greatest) genes identified within the small box. Of all the DEGs detected in this study, the 10 upregulated genes listed in the large box were also the most highly expressed transcripts in response to all three treatments. The DEGs identified within each section (indicated by roman numerals) of the venn diagram are listed in Supplementary Table S2. Numbers below roman numerals indicated the number of DEGs detect in that section. The hyphenated numbers indicate the number of DEGs that were up- or downregulated (up-down) within the total number of DEG identified in that section.

DEGs were then compared between groups to identify common or unique transcripts (Figure 3). The status of expression (up- or downregulated) for all DEGs shared between groups was consistent. For example, *ISG15* was differentially expressed in response to IFNT, IVF-, and AI-derived conceptuses compared to controls and its expression was increased in response to all three groups.

IFNT-dependent differentially expressed genes

Overall, 369 DEGs were found in common between IFNT and AI (19 DEGs) or IFNT, AI and IVF conceptus-exposed endometrium (350 DEGs; Figure 3; Supplementary Table S3), 356 and 12 were up- and downregulated, respectively. We considered these DEGs to be IFNT dependent and related to the maternal recognition of pregnancy signal. Compared to all DEGs, the top 10 most upregulated genes in response to AI and IVF conceptuses were also the 10 most upregulated in response to IFNT and were classical ISGs. Log fold change (logFC) in gene expression was similar across the three groups (Figure 3). The 10 most upregulated genes (greatest to least) in response to conceptuses and IFNT included C-type lectin domain

family 4 member F (*CLEC4F*), *MX2*, radical S-adenosyl methionine domain containing 1 (*RSAD2*), interferon induced protein with tetratricopeptide repeats 3 (*IFIT3*), interferon induced protein 44 (*IFI44*), interferon induced protein with tetratricopeptide repeats 1 (*IFIT1*), interferon induced protein 44 like (*IFI44L*), sterile alpha motif domain containing 9 (*SAMD9*), *ISG15*, and guanylate binding protein 4 (*GBP4*). Conceptuses and IFNT also increased expression of transporters including solute carrier family (SLC) 25, member 28 (*SLC25A28*), *SLC15A3*, *SLC25A30*, *SLC2A6*, and *SLC25A19* as well as *OAS1* and tumor necrosis factor superfamily member 10 (*TNFSF10*; also known as TRAIL) (Supplementary Table S3). Real-time PCR also found that expression of *OAS1*, *TNFSF10*, *ISG15*, and *MX2* was upregulated in the endometrial explants in response to conceptuses and IFNT (Figure 4 and Supplementary Table S4).

Gene ontology analysis of the 356 upregulated IFNT-dependent DEGs identified 135 enriched biological processes ($P \leq 0.05$; Supplementary Table S5) including (1) defense response to virus; (2) type 1 interferon signaling pathway; (3) interferon-gamma-mediated signaling pathway; (4) negative regulation of viral genome replication; and (5) response to virus.

Twelve genes were downregulated by conceptuses and IFNT (Figure 3 and Supplementary Table S3). These genes were associated with six biological processes ($P \leq 0.05$; Supplementary Table S5) including (1) angiogenesis; (2) positive regulation of tyrosine phosphorylation of stat 5 protein; (3) positive regulation of notch signaling pathway; (4) phagocytosis; (5) hemopoiesis; and (6) regulation of cell migration.

Conceptus-induced, IFNT-independent differentially expressed genes

Compared to control endometrium, AI conceptuses altered expression of 179 genes in endometrial explants that were not influenced by IFNT (Figure 5 and Supplementary Table S3). Forty six of these genes were shared with IVF conceptuses; however, 133 were specific to conceptuses produce by AI. Out of the 179 identified genes, 124 and 55 were up- and downregulated, respectively (Supplementary Table S3). The 10 most upregulated genes included (1) trophoblast kunitz domain protein 1 (*TKDP1*); casein 151 (*CSN151*); transmembrane protein 74 (*TMEM74*); zinc finger and SCAN domain containing 10 (*ZSCAN10*), S100 calcium binding protein A8 (*S100A8*); regulator of G-protein signaling 13 (*RGS13*); transmembrane protein 255B (*TMEM255B*); ribosomal protein S15a (*RPS15A*); ubiquitin 8 (*UBQ8*); and recoverin (*RCVRN*) (Figure 5). Amino acid transporters *SLC1A3* and *SLC46A3* were also upregulated by conceptuses but not IFNT (Supplementary Table S3).

The GO analysis of the 124 upregulated genes identified 12 enriched biological processes ($P \leq 0.05$, Supplementary Table S6) including (1) cilium assembly, (2) cilium morphogenesis, (3) DNA double-strand break (DSB) processing, (4) cellular response to DNA damage stimulus and (5) chemokine production, (6) positive regulation of cytokinesis, (7) telomeric 3' overhang formation, (8) cellular response to tumor necrosis factor, (9) negative regulation of telomere capping, (10) cilium movement involved in cell motility, (11) chronic inflammatory response, and (12) DSB repair via nonhomologous end joining.

The 10 most downregulated genes (from most downregulated to least) included (1) multimerin 1 (*MMRN1*), (2) glucagon like peptide 2 receptor (*GLP2R*), (3) immunoglobulin kappa variable 1 (*IGKV1*), (4) calcium voltage-gated channel auxiliary subunit alpha 2 delta 2 (*CACNA2D2*), (5) cholinergic receptor muscarinic 2 (*CHRM2*), (6)

Figure 4. The RT-qPCR relative gene expression of interferon stimulated genes: (A) interferon stimulated gene 15 (*ISG15*), (B) myxovirus resistance protein 2 (*MX2*), and (C) 2'-5'-oligoadenylate synthase 1 (*OAS1*) in endometrium treated with Roswell Park Memorial Institute (RPMI) media (control), ovine interferon tau (IFNT), in vitro fertilization (IVF), and artificial insemination (AI)-derived conceptuses. Data were normalized to the geometric mean expression of peptidylprolyl isomerase A (*PPIA*) and succinate dehydrogenase complex flavoprotein subunit A (*SDHA*) and are presented as least squares means $\pm$ standard error of the least squares means (LSM $\pm$ SEM). Letters over the bars indicate significant differences between treatment means ($P \leq 0.05$).

Figure 5. A venn diagram (Venny 2.1.0 BioinfoGP) identifying DEGs in endometrium treated with ovine interferon tau (IFNT), in vitro fertilization (IVF), and artificial insemination (AI)-derived conceptuses compared to control endometrium. The broken oval circle within the venn diagram indicates genes differentially expressed in response to AI-derived conceptuses but independent of IFNT. The solid oval circle indicates genes differentially expressed in response to IVF but not AI-derived conceptuses. The large broken and solid boxes below the venn diagram include the Ensemble gene ID, gene name, and log fold change (LogFC) in expression (compared to control endometrium) for the 10 most upregulated (greatest to least) and 10 most downregulated (from least downregulated to greatest) genes identified within the corresponding ovals. The DEGs identified within each section (indicated by roman numerals) of the venn diagram are listed in Supplementary Table S2. Numbers below roman numerals indicate the number of DEGs detected in that section. The hyphenated numbers indicate the number of DEGs that were up- or down-regulated (up-down) within the total number of DEGs identified in that section.

cytochrome P450 family 1, member A1 (*CYP1A1*), (7) aquaporin 1 (*AQP1*), (8) tollid like 1 (*TLL1*), (9) ST6 beta-galactoside alpha-2,6-sialyltransferase (*ST6GAL1*), and (10) myozenin 3 (*MYOZ3*) (Figure 5). Out of the DEGs listed above (up- or downregulated), only *AQP1* was shared with the IVF-derived conceptuses (Supplementary Table S3).

The GO analysis of the 55 downregulated genes identified 10 enriched biological processes ($P \leq 0.05$; Supplementary Table S6) including (1) response to wounding, (2) extracellular matrix organization, (3) phosphatidylinositol-mediated signaling, (4) cell adhesion, (5) cell migration, (6) positive regulation of cell migration, (7) phosphatidylinositol phosphorylation, (8) platelet degranulation, (9) protein phosphorylation, and (10) peptidyl-serine phosphorylation.

Expression of interleukin 6 (*IL6*), interleukin 1 beta (*IL1B*), prostaglandin-endoperoxide synthase 2 (*PTGS2*; also known as *COX2*), and nuclear factor kappa B subunit 1 (*NFKB1*), factors involved in inflammatory processes, was measured using RT-qPCR (Figure 6 and Supplementary Table S4). Expression of *IL6* and *IL1B* was affected by treatment (transformed data; $P < 0.05$). Endometrium treated with conceptuses (AI and IVF) had increased expression of *IL6* compared to control endometrium [orthogonal

Figure 6. The RT-qPCR relative gene expression of (A) interleukin 1 beta (*IL1B*), (B) interleukin 6 (*IL6*), and (C) nuclear factor kappa B1 (*NFKB1*) in endometrium treated with Roswell Park Memorial Institute (RPMI) media (control), ovine interferon tau (IFNT), in vitro fertilization (IVF), and artificial insemination (AI)-derived conceptuses. Data were normalized to the geometric mean expression of peptidylprolyl isomerase A (*PPIA*) and succinate dehydrogenase complex flavoprotein subunit A (*SDHA*) and are presented as least squares means $\pm$ standard error of the least squares means (LSM $\pm$ SEM). Letters over the bars indicate significant differences between treatment means ($P \leq 0.05$). The *IL1B* residual data were not normal distributed; therefore, the data were corrected by log transformation (see the statistical analysis section of Materials and Methods); untransformed data are presented in the figure.

contrast 1 (C1), $P < 0.05$]. Compared to IFNT, endometrium treated with conceptuses (AI and IVF) had greater expression of *IL6*, *IL1B*, *NFKB1*, and *PTGS2* [orthogonal contrast 2 (C2), $P \leq 0.05$; Figure 6 and Supplementary Table S4].

IVF conceptus-dependent differentially expressed genes

Compared to control endometrium, however, conceptuses produced by IVF modified expression of 83 genes (60 and 23, up- and down-regulated, respectively) within endometrial explants that were not differentially expressed in response to AI conceptuses (Figure 5 and Supplementary Table S3). Of these DEGs, 22 were also differentially expressed in response to IFNT. Therefore, 61 transcripts were specific to the IVF conceptus treatment only (Figure 5 and Supplementary Table S3). Of the 83 genes for which expression was altered by IVF conceptuses, the 10 most upregulated (greatest to least) were as follows: (1) proprotein convertase subtilisin/kexin type 1 (*PCSK1*), (2) mucin 4 (*MUC4*), (3) leucine rich repeat transmembrane neuronal 2 (*LRRTM2*), (4) tropinin I type 3 interacting kinase (*TNNI3K*), (5) MVP17 mitochondrial inner membrane protein like (*MPV17L*), (6) interleukin 17 (*IL17*), (7) wingless/integrated 93 (*WNT93*), (8) sodium voltage-gated channel alpha subunit 5 (*SCN5A*), (9) family with sequence similarity 26 member F (*FAM26F*), and (10) a disintegrin and metalloproteinase with thrombospondin motifs 8 (*ADAMTS8*) (Figure 5). The a disintegrin and metalloproteinase domain-containing protein 33 (*ADAM33*) and a glycine transporter, *SLC6A9*, were also upregulated by IVF conceptuses when compared to controls (Supplementary Table S3). The GO analysis of upregulated transcripts identified four enriched BP (from greatest to least) including (1) response to wounding, (2) PKR-like ER kinase (PERK)-mediated unfolded protein response, (3) regulation of mRNA stability, and (4) peptide hormone processing ($P \leq 0.05$, Supplementary Table S7).

The 10 most downregulated genes in response to IVF conceptuses (from most downregulated to least) included (1) testis, prostate and placenta expressed (*TEPP*), (2) calcium voltage-gated channel subunit alpha 1 I (*CACNA1I*), (3) protocadherin-12 (*PCDH12*), (4) exocyst complex component 3-like 1 (*EXOC3L1*), (5) double C2-like domain-containing protein beta (*DOC2B*), (6) family with sequence similarity 78 member A (*FAM78A*), (7) leucine zipper protein 1 (*LUZP1*), (8) Usher syndrome type-1C protein-binding protein 1 (*USHBP1*), (9) B-cell CLL/Lymphoma 6B (*BCL6B*), and (10) apoptosis associated tyrosine kinase (*AATK*) (Figure 5). Other genes downregulated and of importance during pregnancy include integrin beta 3 (*ITGB3*), a leukocyte chemokine receptor, C-X-C chemokine receptor 4 (*CXCR4*), and zinc and amino acid transporters, *SLC39A9* and *SLC1A4*, respectively (Supplementary Table S3). The GO analysis of downregulated transcripts identified four enriched BP (from most downregulated to least) that were (1) positive regulation of calcium ion-dependent exocytosis, (2) positive regulation of JUN kinase activity, (3) substrate adhesion-dependent cell spreading, and (4) positive regulation of endothelial cell migration ($P \leq 0.05$; Supplementary Table S7).

Interestingly, in contrast to the comparison of treated explants with the control, a direct comparison of RNAseq data from endometrium treated with AI or IVF-derived conceptuses found no differences in gene expression. This apparent discrepancy is likely related to the variation of expression within each group which will have an effect on the multiple correction of raw P values to determine the FDR, based on which a gene is declared significant or not.

Conceptus sex-dependent differentially expressed genes

A comparison of RNAseq data from endometrial explants exposed to male and female conceptuses with control endometrium resulted in identification of 463 and 638 DEGs, respectively. Of these DEGs, 387 were shared between the two treatments; however, 251 (181 and 70 up- and downregulated) were specific to female and 76 (47 and 29 up- and downregulated) specific to male conceptuses (Figure 7; Supplementary Table S8). The top 10 upregulated DEGs in response to female conceptuses (greatest to least) included (1) *SLC7A3*, (2) proteasome subunit alpha 8 (*PSMA8*), (3) *CSN151*, (4) an unidentified gene (ENSBTAG00000045531), (5) *S100A8*, (6) *TNNI3K*, (7) vomeronasal 1 receptor 4 like (*VN1R4L*), (8) *LRRTM2*, (9) complement factor D (*CFD*), and (10) olfactory receptor 2W1 (*OR2W1*) (Figure 7).

The GO analysis identified 32 enriched BP associated with the 181 upregulated DEGs most of which were associated with some aspect of the immune response ($P \leq 0.05$; Supplementary Table S9). The top five BP included (1) inflammatory response, (2) innate immune response, (3) chemokine production, (4) positive regulation of inflammatory response, and (5) antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent.

The top 10 downregulated transcripts (from most downregulated to least) included (1) *IGKV1*, (2) opioid receptor delta 1 (*OPRD1*), (3) XK-related protein 5 (*XKR5*), (4) myeloproliferative leukemia virus oncogene (*MPL*), (5) *TEPP*, (6) *CHRM2*, (7) *CYP1A1*, (8) *PILR* alpha-associated neural protein precursor (*PIANP*), (9) natriuretic peptide receptor 3 (*NPR3*), and (10) *AQP1* (Figure 7). The GO analysis of the 70 downregulated DEGs (from most downregulated to least) identified 13 enriched BP ($P \leq 0.05$; Supplementary Table S9). The top five downregulated BP included (1) cell migration, (2) chondroitin sulfate biosynthetic process, (3) phosphatidylinositol-mediated signaling, (4) cell adhesion, and (5) marginal zone B-cell differentiation.

The top 10 upregulated DEGs specific to male conceptuses (greatest to least) included (1) *PCSK1*, (2) piwi-like protein 4 (*PIWIL4*), (3) *TKDP1*, (4) transmembrane serine protease 15 (*TMPS15*), (5) *MPV17L*, (6) *SCN5A*, (7) wingless type MMTV integration site family, member 9B (*WNT9B*), (8) *FAM26F*, (9) ribosomal protein L23a (*RPL23A*), and (10) *RPS15A* (Figure 7). There were no enriched BP associated with the DEGs upregulated by male conceptuses. The top 10 downregulated DEGs (from most downregulated to least) included (1) protease, serine 23 (*PRSS23*), (2) B-cell CLL/lymphoma 6 member B protein (*BCL6B*), (3) *SLC16A6*, (4) lysophosphatidic acid receptor 3 (*LPAR3*), (5) trefoil factor 3 (*TFF3*), (6) *SLC5A8*, (7) apelin receptor (*APLNR*), (8) leucine rich repeat containing 15 (*LRRC15*), (9) glycoprotein 1b platelet alpha (*GP1BA*), and (10) ADP-ribosylation factor-like 5C (*ARL5C*) (Figure 7). There were five enriched BP associated with the 29 downregulated DEGs including (1) transmembrane transport, (2) platelet activation, (3) regulation of cell migration, (4) monocarboxylic acid transport, and (5) regulation of small GTPase mediated signal transduction ($P \leq 0.05$; Supplementary Table S10).

A direct comparison of RNAseq data from male and female conceptus-treated endometrium identified six genes that were differentially expressed ($P < 0.001$) in response to conceptus sex (Figures 7 and 8). Expression of 5-hydroxytryptamine receptor 1A (*HTR1A*; LogFC 8.18), *VN1R4L* (LogFC 6.87), myeloid-associated differentiation marker like (*MYADML*; LogFC 4.14), prominin 2 (*PROM2*; LogFC 1.56), and melanophilin (*MLPH*; LogFC 1.01) was greater in endometrium treated with female compared to male conceptuses

Figure 7. A venn diagram (Venny 2.1.0 BioinfoGP) identifying differentially expressed genes (DEGs) in endometrium treated with male and female conceptuses compared to control endometrium. The boxes below the venn diagram include the Ensemble gene ID, gene name, and log fold change (LogFC) in expression (compared to control endometrium) for the 10 most upregulated (greatest to least) and 10 most downregulated (from least downregulated to greatest) genes identified within the corresponding venn diagram section. The DEGs identified within each section (indicated by roman numerals) of the venn diagram are listed in Supplementary Table S6. Numbers below roman numerals indicated the number of DEGs detected in that section. The hyphenated numbers indicate the number of DEGs that were up- or downregulated (up-down) within the total number of DEG identified in that section. The location and direction of change (up- or downregulated) for five of the six DEGs (*MLPH*, *MYADML*, *HTR1A*, *VN1R4L*, *ARL4C*) revealed by a direct comparison of RNA Seq data from endometrium exposed to male vs. female conceptuses is indicated.

Figure 8. Relative gene expression (log fold change, LogFC) in endometrial explants exposed to male vs. female conceptuses for ADP-ribosylation factor-like 4C (*ARL4C*), melanophilin (*MLPH*), prominin 2 (*PROM2*), myeloid-associated differentiation marker like (*MYADML*), vomeronasal 1 receptor 4 like protein (*VN1R4L*), and 5-Hydroxytryptamine receptor 1A (*HTR1A*). Expression of *ARL4C* was greater ($P < 0.001$) in male compared to female conceptus-treated endometrium. Expression of *MLPH*, *PROM2*, *MYADML*, *VN1R4L*, and *HTR1A* was greater ($P < 0.001$) in female compared to male conceptus-treated endometrium.

($P < 0.001$). Specifically, expression of genes *HTR1A* and *VN1R4L* was more than sixfold greater in endometrium treated with female compared to male conceptuses (Figure 8). Expression of a single gene, ADP-ribosylation factor-like 4C (*ARL4C*), was greater (LogFC 1.33) in endometrium treated with male conceptuses ($P < 0.001$). Statistical analysis of the RT-qPCR data suggested a tendency for greater expression ($P = 0.070$) of Lectin, galactose binding, soluble 1 (*LGALS1*; also known as *GAL1*) in endometrium treated with

Figure 9. The RT-qPCR relative gene expression of endometrial Lectin, galactose binding, soluble 1 (*LGALS1*; also known as *GAL1*) in response to Roswell Park Memorial Institute (RPMI) media (control) and male and female conceptuses. Data were normalized to the geometric mean expression of peptidyl-prolyl isomerase A (*PPIA*) and succinate dehydrogenase complex flavoprotein subunit A (*SDHA*) and are presented as least squares means $\pm$ standard error of the least squares means (LSM $\pm$ SEM). Letters over the bars indicate significant differences between treatment means ($P \leq 0.05$). There was a tendency ($P = 0.070$) for an effect of treatment on endometrial expression of *LGALS1*. Male conceptuses induced greater endometrial expression of *LGALS1* compared to female conceptuses.

male compared to female conceptuses (Figure 9 and Supplementary Table S11).

Discussion

This study aimed to identify conceptus-induced modifications to the bovine endometrial transcriptome both dependent and independent

of IFNT, the maternal recognition of pregnancy signal. In addition, comparisons were made between endometrium treated with male or female and AI or IVF-derived conceptuses to identify factors associated with conceptus sex and origin, respectively. Major findings from this study are as follows: (1) identification of novel IFNT-dependent endometrial responsive genes; (2) identification of novel conceptus induced, IFNT-independent endometrial responsive genes; (3) identification of endometrial genes responsive to conceptus origin (AI or IVF); and (4) identification of endometrial genes responsive to conceptus sex in cattle.

In previous studies, endometrial explants were used to model uterine responses to bacteria lipopolysaccharides [30, 44], early embryos [31], and conceptus secretory proteins [45]. In this study, we employed an endometrial explant-conceptus co-culture model to elucidate fine dialog between the early conceptus and endometrium. Endometrial explants can overcome limitations of studying conceptus-maternal interactions. Biopsied explants maintain normal cellular and extracellular architecture and although lacking blood circulation, allow for communication between resident populations of endometrial cells which cannot be achieved with current 2D and 3D cell culture technologies.

RNAseq identified 737 unique transcripts corresponding to 708 identifiable genes in endometrial explants as differentially expressed in response to IFNT and/or early elongating conceptuses (AI or IVF derived) compared to nontreated controls. Approximately two-thirds (67%) of all genes differentially expressed in response to AI conceptuses were IFNT-dependent. The top 10 most upregulated genes in response to AI and IVF-derived conceptuses were classical ISGs and also upregulated in the endometrium during early pregnancy as well as by endometrial cells treated with IFNT [22, 46, 47]. There were an additional 359 IFNT-dependent DEGs shared with AI conceptuses (Supplementary Table S3). Log fold change differences for at least the top 10 most upregulated and downregulated DEGs shared between the IFNT and conceptus treatments were similar (Figure 3).

One-third of all genes differentially expressed in response to an AI conceptus were not shared with the IFNT treatment. In an attempt to identify conceptus-induced, IFNT-independent DEGs in cattle, Bauersachs et al. [20] compared microarray data from endometrium treated with human IFNA to endometrium collected on day 15 and 18 of pregnancy and developed a list of genes potentially influenced by conceptuses, but not a type I IFN. Using RT-qPCR, we assayed expression of nine genes from this list including *NFATC3*, *TDGF1*, *TRAK2*, *MFAP5*, *SLC25A33*, *SLC25A5*, *SLC40A1*, *FABP3*, and *DKK1*. Based on our RT-qPCR and RNAseq data, these genes were not differentially expressed in endometrial explants in response to conceptuses or ovine IFNT (Supplementary Table S4). There are several reasons why this might be so including the different models used (in vivo exposure vs in vitro explants), the use of IFNA vs IFNT, or the use of different technologies (microarray vs RNA-Seq). It is also possible that the conceptus-induced, IFNT-independent DEGs detected in Bauersachs et al. [20] were upregulated in endometrium after prolonged exposure to the conceptus but may not be detected after a 6 h co-culture as used in this study.

Double-strand-break repair protein rad21-like protein 1 (*RAD21L1*) was included in the list from Bauersachs et al. [20] and was identified in this study by RNAseq as a conceptus-induced, IFNT-independent gene. Many of the biological processes associated with genes upregulated by conceptuses but not IFNT included DNA DSB repair mechanisms and telomere maintenance involving double strand break repair protein rad50 (*RAD50*), nibrin (*NBN*), SET do-

main and mariner transposase fusion gene (*SETMAR*), and/or TRAF family member associated nuclear factor kappa B activator (*TANK*).

A complex link exists between inflammation, DNA damage, and DNA repair response mechanisms [48, 49]. Inflammation can elicit DNA damage and induce DNA repair responses including DSB repair [48–50]. A delicate interplay of proinflammatory and anti-inflammatory signaling takes place within the endometrium during early pregnancy in mammals. Conceptuses release cytokines and prostaglandins, many of which are characterized as proinflammatory in other tissues and organs, during initial crosstalk with the endometrium. These factors trigger inflammatory-like microenvironments within the endometrium that involve activation of NFkB signaling pathways [51–53]. In turn, the factors may contribute to uterine histotroph, trophoblast cell motility and adhesion, immune cell activity such as trafficking and vascularity [51]. Chronic inflammatory response was an enriched biological process associated with conceptus-induced, IFNT-independent DEGs and biological processes associated with genes upregulated by AI conceptuses, both dependent and independent of IFNT, were related to inflammation and TNF and NFkB signaling. Activation of inflammatory-like pathways within the endometrium may trigger DNA repair response mechanisms, protecting endometrial cell DNA from degradation. Recently, it was discovered that during mitosis some DSB repair mechanisms have a deleterious effect on cell survival as a result of telomere fusion between chromosomes [54]. It is possible that conceptus induction of genes involved in telomere maintenance within the endometrium help to protect telomeres, possibly from fusion between chromosomes, during activation of DSB repair mechanisms.

Cilium assembly, morphogenesis, and movement were amongst the most highly enriched biological processes associated with conceptus-induced, IFNT-independent, DEGs. Genes involved in these processes included cilia and flagella associated protein 54 (*CFAP54*) and 46 (*CFAP46*), WD repeat domain 19 (*WDR19*; also known as *IFT144*), tubulin tyrosine ligase like 8 (*TTL8*), kinesin family member 27 (*KIF27*), centrosomal protein 126 (*CEP126*; also known as *KIAA1377*), intraflagellar transport 74 (*IFT74*), and tetra-ricopeptide repeat domain 21A (*TTC21A*). Oviduct and uterine epithelial cell cilia have essential functions during reproduction in mammals [55]. Endometrial gene expression signatures of women who experience repeated implantation failure are characterized by reduced expression of genes associated with cilia formation [56]. The endometrium of the ruminant uterus contains aglandular caruncular and glandular intercaruncular regions. The intercaruncular glands have both ciliated and nonciliated epithelial cells and release uterine histotroph, a collection of factors such as ions, lipids, sugars, amino acids, proteins, and extracellular vesicles, secreted and/or transported into the uterine lumen [1]. The histotroph modifies the uterine milieu and is critical for development and elongation of the conceptus during the protracted phase of uterine unattachment. Components of the histotroph are severely reduced and sheep conceptuses fail to elongate in ewes lacking uterine glands [57]. Thus, the conceptus may promote cilia development and movement within the glandular endometrium to promote transport of histotroph into the surrounding microenvironment for nutrition and development of the conceptus.

SLC transporters

The SLC transporters, a group of membrane bound transporter proteins encoded by over 300 genes, transport a wide range of micro- and macromolecules across cell membranes [58]. Expression

of SLC transporters within the endometrium, particularly uterine luminal and glandular epithelial cells, modifies uterine histotroph [59–63]. Five transporters were upregulated by IFNT and bovine conceptuses (AI and IVF-derived) and appear to be IFNT dependent. They included the plasma membrane glucose transporter SLC2A6, the lysosome histidine and peptide transporter SLC15A3, a mitochondrial transporter of unknown solute, SLC25A30, and the mitochondrial iron and the mitochondrial thiamine pyrophosphate transporters SLC25A28 and SLC25A19, respectively. Elevated expression of *SLC15A3* has been reported on day 18 of gestation in bovine endometrium and in bovine endometrial glandular epithelial and stroma cells treated with trophoblast-derived IFNT [59]. There are few data on expression of glucose transporter *SLC2A6* in mammalian reproductive tissues. Expression of *SLC2A6* does increase concomitant with development in Rhesus monkey preimplantation embryos [64]. In sheep, IFNT increased expression of glucose (SLC2A1 and SLC5A1) and arginine (SLC7A2B) transporters in uterine epithelial cells [65]. Indeed, concentrations of glucose and arginine increase in the uterine lumen of sheep between day 10 and 15 of pregnancy and stimulate proliferation, migration, and gene transcription in conceptus trophectoderm cells through activation of the MTOR pathway [65]. Intrauterine glucose concentrations are similar between days 6, 8, and 14 of the estrous cycle in cattle [66]. There are limited data of intrauterine glucose concentrations at the initiation of elongation of the bovine conceptus. As in sheep, bovine conceptus IFNT may increase expression of SLC5A1 within the adjacent endometrium, influencing the availability of glucose to the expanding trophectoderm. Although expression of *SLC5A1* was found to be greater in day 19 pregnant endometrium of dry and lactating dairy cows when compared to inseminated dairy heifers, intrauterine glucose concentrations were similar [67]. Interestingly, in a recent study from our group, glucose infusion into late lactation dairy cows from days 7 to 14 post estrus had an adverse impact on early embryonic development; transfer of day 7 blastocysts to lactating dairy cows reduced mean conceptus length, width, and area on day 14. In addition, a greater proportion of embryos in the control group had elongated to >16 mm in length compared to the glucose infused group [68]. Considering the importance of glucose during development of the ovine conceptus, investigation of intrauterine glucose concentrations during elongation of the bovine conceptus may have practical implications.

A family of transporters (SLC7A1, SLC7A2A, SLC7A2B, and SLC7A3) can transport cationic AA arginine, lysine, and ornithine across the cell plasma membrane [69]. Arginine stimulates trophoblast cell proliferation in both sheep and pigs [70, 71]. Uterine luminal fluid (ULF) concentrations of AA increase during the estrous cycle and are greater in pregnant compared to cyclic heifers on day 18 [72]. Forde et al. [72] measured conceptus and endometrial expression of AA transporters and intrauterine concentrations of AA between cyclic (days 7 to 16) and pregnant (days 7 to 19) cattle. Investigation of arginine and lysine transporters on day 16 found no difference in intercaruncular endometrial expression of *SLC7A1* between cyclic and pregnant heifers. Expression of *SLC7A1* increased with day, however, and was greater in day 19 compared to day 16 pregnant endometrium, coordinate with elevated concentrations of arginine found within pregnant ULF [72]. In cattle, endometrial expression of *SLC7A1* appears to be modulated, at least in part, by P4; cyclic heifers with greater circulating concentrations of P4 have greater endometrial expression of *SLC7A1* [72]. Upregulation of endometrial transporters, including *SLC7A1*, and their activity in heifers with elevated P4 may contribute to the advancement of con-

ceptus growth observed in these animals [72]. In sheep, endometrial expression of *SLC7A1* increases in response to P4 and IFNT [61], also coordinate with increased arginine in the ULF [60]. We did not detect an increase in intercaruncular expression of *SLC7A1* in response to IFNT suggesting either differences in the way this transporter is regulated within the endometrium of cattle and sheep or that *SLC7A1* increases after prolonged stimulation by IFNT (greater than 6 h). Interestingly, expression of *SLC7A3* was greater (5.58; logFC) in endometrium exposed to female but not male conceptuses relative to control media alone (Supplementary Table S6). This was somewhat unexpected because concentrations of arginine and lysine were found to be greater in uteri containing day 19 male compared to female conceptuses [73].

Two AA transporters were upregulated by AI-derived conceptuses alone and may be conceptus-induced, IFNT-independent genes. The upregulated transporters include the glutamate and aspartate transporter SLC1A3, located in the plasma membrane and mitochondria, and a lysosomal transporter of various AA, SLC46A3. Forde et al. [72] found little change in the uterine lumen concentration of glutamate and aspartate before day 16 of pregnancy; however, concentration of both AA increases between day 16 and 19 of gestation. Acidic AA transporters SLC1A1, -1A2, and -1A3 transport glutamate and aspartate while SLC1A4 and SLC1A5 transport glutamate and/or neutral AA such as the glutamate and aspartate derivatives glutamine and asparagine, respectively. Forde et al. [72] detected an increase in *SLC1A5* expression in pregnant intercaruncular endometrium between days 13 and 16 of gestation suggesting that this transporter may be partially responsible for the intrauterine increase in glutamate and aspartate. Similar to transporter SLC7A1, pregnant heifers with greater circulating concentrations of P4 and larger conceptuses have an earlier increase in endometrial expression of *SLC1A5* suggesting a possible link between SLC1A5, elevated concentrations of glutamate/aspartate, and advanced conceptus development [72]. Interesting, IVF-derived conceptuses did not increase endometrial *SLC1A3* expression over controls and downregulated endometrial expression of *SLC1A4*.

The IVF-derived conceptuses alone upregulated and downregulated the plasma membrane sodium and chloride-dependent neurotransmitter glycine transporter 1 (*SLC6A9*) and the zinc transporter *SLC39A9*, respectively, compared to controls. Glycine, an osmolyte and essential precursor for synthesis of proteins and nucleic acids, is one of the most abundant AA found within the uterine lumen of both sheep and cattle [72]. Zinc is an essential mineral for cellular catalytic and signaling processes involving hundreds of different enzymes and proteins [74]. Data in rodents suggest that zinc is important pre- and postconception for early embryonic development. A preconception zinc deficiency in rodents results in decreased embryo length and a high incidence of pregnancy loss [74]. Furthermore, murine embryos cultured under low zinc conditions have abnormal morphology and a reduction in extraembryonic endoderm [75]. Little is known about bovine conceptus glycine or zinc requirements during elongation; however, addition of zinc or glycine to bovine in vitro embryo production systems may improve pre-implantation embryo development [76, 77].

Chemokines

The CC and CXC chemokine family of ligands function through seven-transmembrane, G protein-coupled receptors (CCR or CXCR) to regulate angiogenesis, leukocyte chemotaxis, hematopoiesis, immune surveillance, and initiation of adaptive immune responses [78].

The chemokines and their receptors are expressed within the reproductive tissues of mammals and are believed to regulate essential functions during establishment of pregnancy. Both IFNT and conceptus treatments increased endometrial expression of chemokines *CCL8*, *CXCL9*, *CXCL10* (also known as IP-10), and *CXCL11* in this study. In cattle, an increase in endometrial *CCL8* and *CXCL10* during early pregnancy or in response to bovine IFNT was reported recently [79]. In sheep, expression of *CXCL9*, *CXCL10*, and *CXCL11* increases within the endometrium during the peri-implantation period and treatment of day 14 cyclic caruncular endometrium with IFNT or IFNG increases caruncular *CXCL9* expression [80]. Interferon stimulation of *CXCL9*, *CXCL10*, and *CXCL11* expression, all of which bind the same chemokine receptor, CXCR3, may function to regulate endometrial immune cell and/or trophoblast chemotaxis [80, 81]. Culture media collected from sheep caruncles stimulated with bovine IFNT increased peripheral blood leukocyte chemotaxis, a response blocked after the addition of an anti-CXCL10 antibody [81].

Conceptuses produced by IVF decreased endometrial expression of the chemokine receptor CXCR4. Investigations of endometrial CXCR4 and its ligand, CXCL12, in women and sheep suggest these factors have importation functions related to uterine receptivity, maternal-conceptus immune tolerance and angiogenesis. Endometrial expression of CXCR4 and CXCL12 increases during the receptive phase in women and sheep, respectively [82–84]. In ruminants, evidence suggests that CXCR4 and CXCL12 coordinate placental angiogenesis. Ovine trophoblast cells treated with CXCL12 increase expression of angiogenic factors VEGF and FGF2 and osmotic pump release of a CXCR4 antagonist into the uteri of pregnant sheep decreases endometrial and fetal membrane VEGF protein [82, 83]. The role of endometrial CXCR4 has not been elucidated in cattle.

Conceptus sex

Data from studies investigating endometrial responses to conceptus sex in cattle are somewhat conflicting. Forde et al. [73] compared ULF-AA abundance and endometrial expression profiles of bovine uteri carrying day 19, male or female conceptuses. The ULF-AA profiles differed; however, differences in endometrial expression were not detected. In two separate studies by Gómez et al. [85, 86], conceptus sex altered intrauterine protein content and cultured endometrial cell gene expression by day 8 of development [85, 86]. In the current study, RNAseq identified 251 and 76 DEGs in endometrium treated specifically with female and male conceptuses, respectively, compared to controls. A direct comparison of endometrium cultured with male or female conceptuses identified six differentially expressed genes in the present study.

Endometrial expression of *ARL4C*, a member of the small GTP binding protein family, was greater in response to male conceptuses compared to females. Recent studies suggest the *ARL4C* binds alpha tubulin and is involved in lipid metabolism, epithelial cell morphogenesis, and tubulogenesis resulting in lumen formation [87, 88]. *ARL4C* expression increases in response to Wnt-beta-catenin and growth factor-Ras-mitogen-activated protein kinase signaling [87, 88]. Deregulation of Ras or *ARL4C* leads to inflammatory disorders and tumor formation possibly through uncontrolled epithelial cell outgrowth [87–89]. Interestingly, a member of the Wnt family, Wnt9B, was upregulated (LogFC 2.78) in endometrium in response to male but not female conceptuses compared to controls in this study. Furthermore, BP and KEGG pathways associated with genes downregulated by male conceptuses compared to controls included

regulation of small GTPase mediated signal transduction and the Ras-associated protein-1 (Rap1) signaling pathway, respectively. Activated Rap1, a small GTPase, can negatively regulate Ras activity. The role of endometrial *ARL4C* and its significance in establishment of pregnancy is unknown.

Exposure of endometrial explants to a female conceptus up-regulated expression of five genes (*MLPH*, *PROM2*, *MYADM*, *VN1R4L*, and *HTR1A*) compared to endometrium exposed to male conceptuses. These factors have not been investigated or reported during early pregnancy in cattle or other mammals. The protein *MLPH* forms a tripartite complex with proteins Ras-related protein RAB27A and Myosin-Va to distribute melanosomes, melanin containing organelles, within melanocytes resulting in skin, eye, and hair pigmentation [90]. During the receptive phase, endometrial *MLPH* was found to be differentially expressed in women who had elevated levels of P4 during the prior follicular phase [91].

The protein *PROM2* is a member of the prominin family of pentaspan membrane glycoproteins and has been localized to the basal membrane of epithelial cells. *PROM2* interacts with cholesterol and may be involved in the organization of plasma membrane protrusions and microdomains such as cilia, microvilli, and extracellular vesicles and was found within plasma membrane particles retained within cell culture media or released in urine [92].

Expression of *MYADM* was first discovered in multipotent progenitor cells and is upregulated during myeloid cell differentiation [93]. The *MYADM* protein has also been localized within epithelial cell membrane protrusions and may play a role in cell migration through development of lamellipodium [94]. A recent study of bovine endometrial fibroblasts found that *MYADM* protein could be localized within fibroblast exosomes when cells were cultured in reduced oxygen [95].

Expression of a vomeronasal 1 receptor 4 like protein (*VN1R4L*) was nearly sevenfold greater in endometrium cultured with female compared to male conceptuses. The role of endometrial vomeronasal 1 receptors (V1R), a family of receptors for intraspecies chemosignals, such as pheromones, is unknown. The V1R family consists of approximately 34 homologs in cattle and is typically expressed in olfactory epithelium and the vomeronasal organ [96].

Expression of *HTR1A*, a serotonin receptor, was more than eightfold greater in endometrium cultured with female compared to male conceptuses. The *HTR1A* protein is widely expressed within the mammalian brain and its activation negatively regulates the serotonin system [97]. Expression of *HTR1A* has been detected within ectopic endometrial tissue of women and is upregulated in ovarian endometriosis compared to eutopic endometrial samples [98]. Serotonin is biochemically derived from tryptophan and controls many physiological systems including gastrointestinal motility and secretion, hemostatic processes, circadian rhythms, and behavior. Serotonin has been found within reproductive tissues of rodents, including the ovary, oviduct, uterus, and placenta, and it believed to signal locally to regulate embryonic and fetal development [99]. Preimplantation mouse embryos express serotonin receptors and targeted disruption of maternal tryptophan hydroxylase 1 (TPH), the rate-limiting enzyme involved in serotonin synthesis, results in offspring with abnormal central nervous system development [100, 101].

L-Tryptophan can be converted to 5-hydroxytryptophan (serotonin) by TPH followed by the activity of aromatic L-amino acid decarboxylase (DOPA decarboxylase). On the other hand, L-Tryptophan may also be metabolized along the kynurenine pathway, by an initial enzymatic step involving tryptophan 2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenase 1 (IDO1),

and/or IDO2 enzymes. In pregnant heifers, intrauterine tryptophan increases as pregnancy progresses [59, 72] and is greater within the uterine lumen of male vs. female pregnancies on day 19 of gestation [73]. However, endometrial L-tryptophan decreases coincident with an increase in endometrial *IDO1* expression and L-kynurenine abundance [102]. Upregulation of *IDO1* in pregnant endometrium appears to be the result of conceptus IFNT. Groebner et al. [102] found that *IDO1* expression increased in cultured endometrial fibroblast treated with IFNT. In this study, *IDO1* was detected as a conceptus (AI and IVF-derived) induced, IFNT-dependent endometrial DEG consistent with findings by Groebner et al. [102]. Importantly, metabolites produced by the enzymatic action of IDO on tryptophan contribute to immune suppression in the tumor microenvironment and at the fetal–maternal interface in mammals contributing to fetal–maternal immune tolerance [103]. To the best of our knowledge, it is not known if TPH is expressed within bovine conceptus or endometrium or if serotonin is present to act on endometrial HTR1A during female pregnancies. It is possible that some tryptophan is converted to serotonin within the female conceptus microenvironment to act on endometrial HTR1A. However, further investigation of the HTR1A-serotonin system during early pregnancy in cattle will clarify this and enhance our understanding of endometrial modifications that are dependent on conceptus sex.

In conclusion, these data add to a growing body of evidence in sheep and cattle demonstrating that conceptus-derived factors other than IFNT may have a biological role in pregnancy establishment. These factors may include prostaglandins and cortisol, which can modify the endometrium both alone and in tandem with IFNT in addition to conceptus-derived proteins which are detectable in uterine lumen fluid from heifers on day 16 of pregnancy [26, 46, 104, 105]. The data are consistent with those of Moraes et al. [11] whose findings from the transcriptome analyses of the endometrium support the idea that conceptus–endometrial interactions are dysregulated in subfertile heifers and may disrupt normal implantation and placentation, leading to pregnancy loss.

Supplementary data

Supplementary data are available at [BIOLRE](https://doi.org/10.1016/j.biolre.2019.01.010) online.

Supplementary Table S1. Summary data showing length (mean $\pm$ SEM) and sex of day 15 conceptuses cultured on top of endometrial explants.

Supplementary Table S2. GenBank accession number, gene name, primer direction, primer sequence, product size, and percent amplification efficiency of cDNAs amplified during real-time quantitative-polymerase chain reaction (RT-qPCR).

Supplementary Table S3. List of up- and down regulated endometrial DEGs identified when comparing ovine interferon tau (IFNT) and in vitro fertilization (IVF) and artificial insemination (AI)-derived conceptus treatments in the venn diagram presented in Figure 2.

Supplementary Table S4. Relative expression values of target genes in response to RPMI media (control), IFNT and IVF and AI-derived day 15 bovine conceptuses. Relative expression data were normalized over the geometric mean of reference genes *PPIA* and *SDHA*. Data are presented as least squares means $\pm$ standard error of the least squares means (LSM $\pm$ SEM). Letters indicate significant differences between treatments.

Supplementary Table S5. Up- and downregulated biological processes (BP) and Kyoto encyclopedia of genes and genomes (KEGG)

pathways in bovine endometrium that were associated with up- and downregulated interferon tau (IFNT) dependent genes (356 and 12, respectively). The genes were differentially expressed in response to ovine interferon tau (IFNT) and conceptus treatments (AI and IVF). There were no KEGG pathways associated with the downregulated IFNT-dependent differentially expressed genes (DEGs).

Supplementary Table S6. Up- and downregulated biological processes (BP) and Kyoto encyclopedia of genes and genomes (KEGG) pathways in bovine endometrium that were associated with up- and downregulated conceptus-induced interferon tau (IFNT) independent genes (144 and 55, respectively).

Supplementary Table S7. Up- and downregulated biological processes (BP) and Kyoto encyclopedia of genes and genomes (KEGG) pathways in bovine endometrium that were associated with up- and downregulated in vitro fertilization (IVF) conceptus-dependent genes (60 and 23, respectively). There were no KEGG pathways associated with the IVF conceptus-dependent genes.

Supplementary Table S8. List of up- and downregulated endometrial differentially expressed genes (DEGs) identified when comparing male and female conceptus treatments in the venn diagram presented in Figure 6.

Supplementary Table S9. Up- and downregulated biological processes (BP) and Kyoto encyclopedia of genes and genomes (KEGG) pathways in bovine endometrium that were associated with up- and downregulated female conceptus-induced genes (181 and 70, respectively). There were no KEGG pathways associated with female-induced downregulated differentially expressed genes (DEGs).

Supplementary Table S10. Downregulated biological processes (BP) and Kyoto encyclopedia of genes and genomes (KEGG) pathways in bovine endometrium that were associated with downregulated male conceptus-induced genes (47 and 29, respectively). There were no BP or KEGG pathways associated with the male induced upregulated genes.

Supplementary Table S11. Relative expression values of target genes in response to RPMI media (control), male or female day 15 bovine conceptuses. Relative expression data were normalized over the geometric mean of reference genes *PPIA* and *SDHA*. Data are presented as least squares means $\pm$ standard error of the least squares means (LSM $\pm$ SEM). Letters indicate significant differences between treatments (Trt).

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