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Conceptus-Induced Changes in the Endometrial Transcriptome: How Soon Does the Cow Know She Is Pregnant?¹

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ABSTRACT

This study sought to determine the earliest response of the bovine uterine endometrium to the presence of the conceptus at key developmental stages of early pregnancy. There were no detectable differences in gene expression in endometria from pregnant and cyclic heifers on Days 5, 7, and 13 postestrus, but the expression of 764 genes was altered due to the presence of the conceptus at maternal recognition of pregnancy (Day 16). Of these 514 genes, *MX2*, *BST2*, *RSAD2*, *ISG15*, *OAS1*, *USP18*, *IFI44*, *ISG20*, *SAMD9*, *EIF4E*, and *IFIT2* increased to the greatest extent in pregnant endometria (>8-fold log₂ fold change increase). The expression of *OXTR*, Bt.643 (unofficial symbol), and *KCNMA1* was reduced the most, but short-term treatment with recombinant ovine interferon tau (IFNT) in vitro or in vivo did not alter their expression. In vivo intrauterine infusion of IFNT induced the expression of *EIF4E*, *IFIT2*, *IFI44*, *ISG20*, *MX2*, *RSAD2*, *SAMD9*, and *USP18*. These results revealed for the first time that changes that occur in the endometrial transcriptome are independent of the presence of a conceptus until pregnancy recognition. The differentially expressed genes (including *MX2*, *BST2*, *RSAD2*, *ISG15*, *OAS1*, *USP18*, *IFI44*, *ISG20*, *SAMD*, and *EIF4E*) are a consequence of IFNT production by the conceptus. The identified genes represent known and novel early markers of conceptus development and/or return to cyclicity and may be useful to identify the earliest stage at which the endometrial response to the conceptus is detectable.

bovine, female reproductive tract, gene expression, interferon tau, pregnancy

INTRODUCTION

In ruminants, implantation is a prolonged process characterized by elongation of the hatched blastocyst followed by apposition, attachment, and adhesion of the trophoctoderm to the uterine luminal epithelium (LE) [1, 2]. The process of conceptus elongation posthatching is regulated by histotroph-

derived factors, as evidenced by the fact that elongation does not occur in the absence of uterine glands [3] and that despite attempts to artificially induce this process [4, 5], hatched bovine blastocysts fail to elongate normally in vitro but will do so if transferred to the uterus of a recipient female at the correct stage of the estrous cycle [6].

Therefore, the uterine endometrium plays a central role in the context of early conceptus-maternal communication for establishment and maintenance of pregnancy. This involves dynamic changes in the uterine epithelia that are tightly regulated by changes in steroid hormones, cytokines, and growth factors and their receptors. These factors help establish receptivity of the uterine LE to the developing conceptus and play a key role in regulating differentiated functions of the uterine glandular epithelium (GE), which is required for histotroph secretion and stromal cells, which are required for protection of the developing semiallograph conceptus from the maternal immune system [7].

During the peri-implantation period of pregnancy, endometrial gene expression is regulated primarily by the sequential actions of maternally derived progesterone and conceptus secretions, principally interferon tau (IFNT), and the expression patterns of these genes are regulated by either progesterone alone, IFNT alone, or the actions of both [8]. In cattle, pregnancy recognition must occur by Day 16 [9, 10], with sufficient secretion of IFNT from the conceptus trophoctoderm to inhibit the production of luteolytic pulses of prostaglandin F_{2α} (PG) by the endometrium [11–13]. Key molecular events that occur in the window of time prior to and during the process of maternal recognition of pregnancy are of critical importance to help elucidate the potential pathways that may be responsible for conceptus survival. A number of studies have examined mRNA expression profiles of bovine oviductal epithelial cells [14, 15] or endometrium [16] at estrus (low progesterone) and at diestrus (high progesterone) periods and around the peri-implantation period of pregnancy [17–19]. Recent studies found that embryos of differing developmental fates may elicit different responses from the endometrium [20] and that the endometrial transcriptome profile around the time of initiation of implantation (Days 18–20) may be predictive of the ability of the conceptus to maintain pregnancy [21]. However, to date, there has been no comprehensive transcriptional profiling of bovine endometrial gene expression at key developmental stages of early pregnancy. Indeed, no studies have examined endometrial gene expression in cattle earlier than pregnancy recognition, despite the fact that the period of greatest reproductive wastage occurs before Day 16 [11, 22].

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Using a combination of *in vivo* and *in vitro* models, studies were conducted to 1) identify the response of the uterine endometrium in terms of alterations to the transcriptome to the presence of a conceptus at key developmental stages of early pregnancy; and 2) to determine the contribution of IFNT to the regulation of these differentially expressed genes (i.e., whether these genes are directly induced or indirectly regulated by IFNT).

MATERIALS AND METHODS

Experiment 1: Identification of the Transcriptional Changes in the Endometrium at Key Developmental Stages of Early Pregnancy

The aim of this experiment was to establish a comprehensive transcriptome profile of bovine endometrial gene expression at key developmental stages of early pregnancy leading up to maternal recognition of pregnancy by comparing the transcriptome of endometria from pregnant and cyclic heifers.

Animals and Treatments

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland, 1897) and the European Community Directive 86/609/EC and were sanctioned by the Animal Research Ethics Committee of University College Dublin. The experimental design used has been described previously [23]. Briefly, cross-bred beef heifers ($n = 263$) were synchronized by insertion of a controlled internal drug release device (1.94 g of progesterone; InterAg, Hamilton, New Zealand) placed into the vagina for 8 days. A 0.5-mg intramuscular injection of a PG analog (PG, Estrumate, Shering-Plough Animal Health, Hertfordshire, U.K.) was administered 3 days before controlled internal drug release device removal. Heifers were checked for standing estrus and only those exhibiting estrus (Day 0) within a narrow time frame were used. Heifers were assigned randomly to be either inseminated ($n = 140$) or to remain noninseminated cyclic controls ($n = 70$). Heifers in the inseminated group were artificially inseminated with semen from a proven sire at 12 h after onset of estrus. Within these two groups, one-half of the heifers received a progesterone-releasing intravaginal device (containing 1.55 g of progesterone; CEVA Animal Health Ltd, Chesham, U.K.) on Day 3 of pregnancy/estrous cycle to increase circulating concentrations of progesterone [23]. All heifers were subsequently slaughtered on either Day 5, 7, 13, or 16, corresponding, in pregnant heifers, to the 16-cell/early morula stage, blastocyst stage, initiation of conceptus elongation, and elongated filamentous conceptus, respectively. Within 30 min of slaughter, uteri of all heifers were flushed with 20 ml of PBS containing 5% fetal calf serum, and pregnancy was confirmed by the presence of an apparently normal conceptus at the correct stage of development, identified visually using a stereomicroscope. To avoid any potential ambiguity, inseminated heifers from which no conceptus was recovered or the embryo was not at the correct developmental stage were excluded from the experiment. See the study by Carter et al. [23] for precise data on embryo recovery and serum progesterone concentrations. Results from analyses of data for the specific effects of elevated progesterone on the endometrial transcriptome in pregnant heifers were published previously [24].

Tissue Recovery, RNA Isolation, and Microarray Hybridization

The uterine horn ipsilateral to the corpus luteum (CL) was opened longitudinally, and strips of endometrium containing both caruncular and intercaruncular tissue were carefully removed and processed as previously described [24]. Briefly, endometrial samples were immersed in a 1:5 w/v solution of RNAlater (Sigma, Dublin, Ireland), transported to the laboratory on ice, and stored at 4°C for 24 h. Excess RNAlater was removed, and the tissue was stored at -80°C prior to RNA extraction. Total RNA was extracted ($n = 5$ animals per treatment per time point; $n = 80$ in total) using TRIzol reagent (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. RNA cleanup and on-column DNase digestion were performed using the Qiagen mini-kit (Qiagen, Crawley, West Sussex, U.K.). Quality and quantity of the RNA were determined using the Agilent bioanalyzer, and all samples used had an RNA integrity number greater than 8.5 (Agilent Technologies, Santa Clara, CA). Transcriptomic analysis of the uterine endometrium was carried out using the Affymetrix GeneChip Bovine Genome Array, and all microarray protocols were carried out by Almac Diagnostics Ltd. (Craigavon, Armagh, Northern

Ireland) as previously described, using one cycle target labeling [24]. Briefly, 2 µg of total RNA was converted to cDNA via first and second strand synthesis by 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. cRNA quality was assessed on the Agilent 2100 bioanalyzer, and 25 µg of this cRNA was fragmented using 5× fragmentation buffer in RNase-free water contained within the GeneChip Sample cleanup module. The fragmentation reaction was carried out at 94°C for 35 min to generate 35–200 base fragments for hybridization, and fragmented cRNA quality was also assessed using the Agilent bioanalyzer. Fifteen micrograms of fragmented cRNA and the 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. A total of 80 microarrays were analyzed, each representing endometrial tissue from an individual animal, which encompassed two P4 treatments (high and normal P4), two pregnancy states (pregnant or nonpregnant cyclic controls), and four time points (Days 5, 7, 13, and 16 of early pregnancy/estrous cycle) with five animals per treatment per time point.

Data Analyses

The raw data intensity files were read into R software (<http://www.r-project.org/>) and preprocessed using functions of the Affymetrix and Gene Chip Robust Multiarray Average software of the BioConductor project (<http://www.bioconductor.org/>) [25]. Identification of differentially expressed genes (DEGs) in the endometria of pregnant and cyclic heifers was achieved using Limma software using linear modeling and an empirical Bayes framework to shrink the variance of measurements on each probe set. A modified *t*-test was carried out and *P* values were adjusted for multiple testing using the Benjamini and Hochberg false discovery rate method. Lists of DEGs were selected on the basis of an adjusted *P* value of <0.05. A more stringent level of *P* < 0.01 was used to generate the lists of differentially expressed probe sets input for the gene ontology (GO) overrepresentation analysis.

GO Analysis

Analysis of the GO terms was performed using GOstats [26] software of Bioconductor. The chip probes were first filtered as outlined in the GOstats vignette or help page in Bioconductor. This provided a "gene universe" that represented the set of expressed genes across the experimental conditions. Filtering reduced the number of false positives resulting from the analysis, i.e., GO terms marked as statistically significant when in truth they are not. For each list of DEGs identified from different comparisons, e.g., pregnant versus cyclic on Day 16, a conditional hypergeometric statistical test was performed using a cutoff *P* value of 0.01. This selected the overrepresented GO nodes, i.e., those more associated with the probe list than would be expected by chance based on the gene universe while taking into account the structural relationship between GO terms in the GO graph.

Pathway and Interaction Network Analysis

Ingenuity pathway analysis (IPA; <http://www.ingenuity.com>) was carried out for each list of DEGs generated from each of the different comparisons. This allowed the data to be interrogated to determine the functions and/or pathways associated with each of the gene lists and to identify which molecules from a given list of DEGs interacted with one another. To identify the pathways that were associated with each list of DEGs, a *P* value for a given function was calculated by considering the number of functional analysis molecules that participate in that function and the total number of molecules that are known to be associated with that function/pathway in the Ingenuity knowledge base. Functions and pathways with *P* value of less than 0.05 were considered significant for a given list of DEGs. The ratio indicates the number of genes for a particular pathway/ontology in a gene list compared to the total number of genes in a given pathway.

Interaction networks for each list of DEGs were generated by identifying genes that serve as molecules of interest that interact with other molecules in the Ingenuity knowledge base. These serve as "seed" molecules to generate a network with a limit of 35 molecules for each interaction network generated. The networks generated were scored based on the number of network-eligible molecules they contained. The network score was based on the hypergeometric distribution and was calculated using a right-tailed Fisher exact test. The higher the score for a given interaction network, the lower the probability of finding the observed number of network-eligible molecules in a given network by chance.

Real-Time Quantitative PCR

Real-time quantitative PCR (Q-PCR) was used to validate the findings of the microarray data. Briefly, 5 µg of RNA used for microarray analysis was converted to complementary DNA (cDNA) using Superscript III (Invitrogen) and random hexamers per the manufacturer's instructions. All primers were designed using Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primer-blast/). Each real-time Q-PCR assay was carried out on the 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA) with 50 ng of cDNA, optimized primer concentrations (see Supplemental Table S1, available online at www.bioreprod.org), 10 µl of SYBR Green Mastermix (Applied Biosystems) in a final reaction mixture volume of 20 µl. Cycling conditions were as follows: 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, and were carried out with the inclusion of a dissociation curve to ensure specificity of amplification. A standard curve was included for each gene of interest as well as for the normalizer gene to obtain primer efficiencies. Analysis of transcript abundance was determined by the standard curve method, by comparing the gene of interest to the reference gene (*RPL19*). Significance was determined using PROC MIXED model in SAS software (SAS Institute Inc., Cary, NC) with day, pregnancy status, P4 treatment, and their interactions included as the main effects.

Experiment 2: Identification of Genes Directly Induced, or Indirectly Regulated, by IFNT In Vitro

The aim of this experiment was to determine if the DEGs identified in experiment 1 were regulated by IFNT. Primary cultures of endometrial fibroblasts or GE cells were derived and then treated with IFNT (see below). When the regulation occurred within 2 h or at 24 h, the gene was considered an IFNT-induced gene or an IFNT-regulated gene, respectively [18].

Primary Culture of Endometrial Fibroblast and Glandular Epithelial Cells Treated with IFNT

In vitro culture of bovine endometrial cells (fibroblast and glandular epithelial cells only) was carried out as previously described [18]. Briefly, fibroblast and glandular epithelial cells were derived from cyclic cows ($n = 2$) during the luteal phase of the estrous cycle. The endometrium was dissected from the ipsilateral uterine horn and washed in Hanks solution, minced, and dispersed with collagenase and hyaluronidase in Earle buffer saline solution containing 15 mM HEPES and 2% bovine serum albumin for 2 h at 37°C. Clumps of uterine glands were isolated using a strainer, and these GE cell suspensions were seeded into dishes. The fibroblast cells were resuspended in culture medium, seeded in Primaria dishes (VWR, Fontenay-sous-Bois, France) and incubated for 2 h in 20% CO₂. Following preculture, floating cells were carefully eliminated by washing, and 24 h later the adherent fibroblasts were treated with trypsin (TrypLE Express; GIBCO-Invitrogen, Cergy-Pontoise, France) and seeded. Both fibroblasts and GE cells were cultured in Dulbecco modified Eagle medium-F12 medium supplemented with 10% (vol/vol) fetal calf serum (GIBCO-Invitrogen), insulin (Ac-trapid-Novo; Novo-Nordisk, La Defense, France), and penicillin and streptomycin (Sigma). Cultures were maintained at 37°C and 5% CO₂, with medium replaced every 2 days until the preconfluence stage was reached.

Once the preconfluence stage was reached, both fibroblasts and GE cells were starved of serum for 24 h, and cells prepared from each individual cow were treated with 100 ng/ml or 1 µg/ml recombinant ovine IFNT for 30 min, 2 h, or 24 h as previously described [18]. Control cells were left untreated and were recovered at each time point. All cells were recovered by washing with ice cold PBS and scraped and centrifuged at 10 000 rpm at 4°C for 2 min to remove the supernatant. Cell pellets were stored at -80°C until processed for gene expression analysis as reported above with minor modifications. Each real-time Q-PCR assay was carried out on the 7500 Fast real-time PCR system (Applied Biosystems) with 50 ng of cDNA, optimized primer concentrations, and 7.5 µl of SYBR Green Mastermix in a final reaction mixture volume of 15 µl.

Experiment 3: In Vivo Confirmation That Genes Identified as Differentially Expressed During Early Pregnancy Are Due to IFNT Produced by the Conceptus

The aim of this experiment was to confirm in vivo that the DEGs identified in experiment 1 and validated in experiment 2 were specifically induced by IFNT in the endometrium and in what region of the endometrium this occurred (caruncular or intercaruncular).

Infusion of IFNT to the Uterine Endometrium In Vivo

Beef cows were synchronized and observed for standing estrus as described in Mansouri-Attia et al. [18]. Following estrous synchronization, only those heifers observed in standing estrus were utilized further. On Day 14 of the estrous cycle, these nonpregnant cows received a single intrauterine infusion (25 ml/horn) of 0.9% NaCl solution containing recombinant ovine IFNT (200 µg/ml) or vehicle as previously described [27]. Five animals from each group were slaughtered 2 h postinfusion. Caruncles and intercaruncular regions were carefully dissected from each injected horn separately, snap-frozen in liquid nitrogen, and then stored at -80°C until processing for real-time Q-PCR analysis.

RESULTS

Conceptus-Induced Changes in Endometrial Gene Expression Only Become Evident at the Time of Maternal Recognition of Pregnancy

Correspondence analysis, which determines the principal factors that contribute to overall changes in gene expression in a data set, revealed that the source of greatest variation in endometrial gene expression was the day of the estrous cycle and early pregnancy. Samples recovered from heifers on Days 5 and 7 clustered together, and samples recovered on Days 13 and 16 clustered together (Fig. 1A). In addition, samples recovered from Days 5 and 7 exhibited greater variation in their overall transcriptional profile than those recovered at later stages (Fig. 1A). When the pregnancy status of each heifer was taken into account, there was no segregation between pregnant and cyclic heifers on Days 5, 7, or 13; however, on Day 16, endometria from pregnant heifers clustered together and apart from endometria from cyclic heifers (Fig. 1B).

Comparison of endometria from pregnant and cyclic heifers on Days 5, 7, and 13 postestrus ($n = 10$ pregnant and $n = 10$ cyclic heifers per time point) revealed no detectable differences in gene expression. However on Day 16, the presence of a conceptus altered the expression of 764 genes ($P < 0.05$; see Supplemental Table S2) compared to nonpregnant heifers, of which 233 genes were altered by a log₂ fold change of >2 . Of the 514 genes up-regulated in pregnant endometria, myxovirus (influenza virus) resistance 2 (mouse) (*MX2*), similar to bone marrow stromal cell antigen 2 (*BST2*), radical *S*-adenosyl methionine domain containing 2 (*RSAD2*), ISG15 ubiquitin-like modifier (*ISG15*, previously known as *UCRP*), 2',5'-oligoadenylate synthetase 1, 40/46 kDa (*OAS1*), ubiquitin specific peptidase 18 (*USP18*), interferon-induced protein 44 (*IFI44*), interferon stimulated exonuclease gene 20 kDa (*ISG20*), sterile alpha motif domain containing 9 (*SAMD9*), Homo sapiens eukaryotic translation initiation factor 4E (*EIF4E*), transcript variant 2, mRNA, and interferon-induced protein with tetratricopeptide repeats 2 (*IFIT2*) were increased by the greatest amount (>8 -fold increase), while the expression of oxytocin receptor (*OXTR*), potassium large conductance calcium-activated channel, subfamily M, alpha member 1 (*KCNMA1*) as well as a number of genes with unknown function (*Bt.643.1.S1_at*, *Bt.15298.1.A1_at*, *Bt.16721.1.A1_at*, and *Bt.18820.1.S1_at*) decreased by more than 2.5-fold.

Real-Time Q-PCR Validation of Microarray Data

Twelve genes, whose expression was altered to the greatest extent due to pregnancy on Day 16, were chosen for validation. There was a significant effect of day on the expression of *BST2*, *Bt643*, *EIF4E*, *ISG20*, *KCNMA1*, *MX2*, *OAS1*, *OXTR*, *RSAD2*, *SAMD9*, and *USP18* (Fig. 2; $P < 0.05$). Of the 12 genes validated, pregnancy status had a significant effect of the

(A)

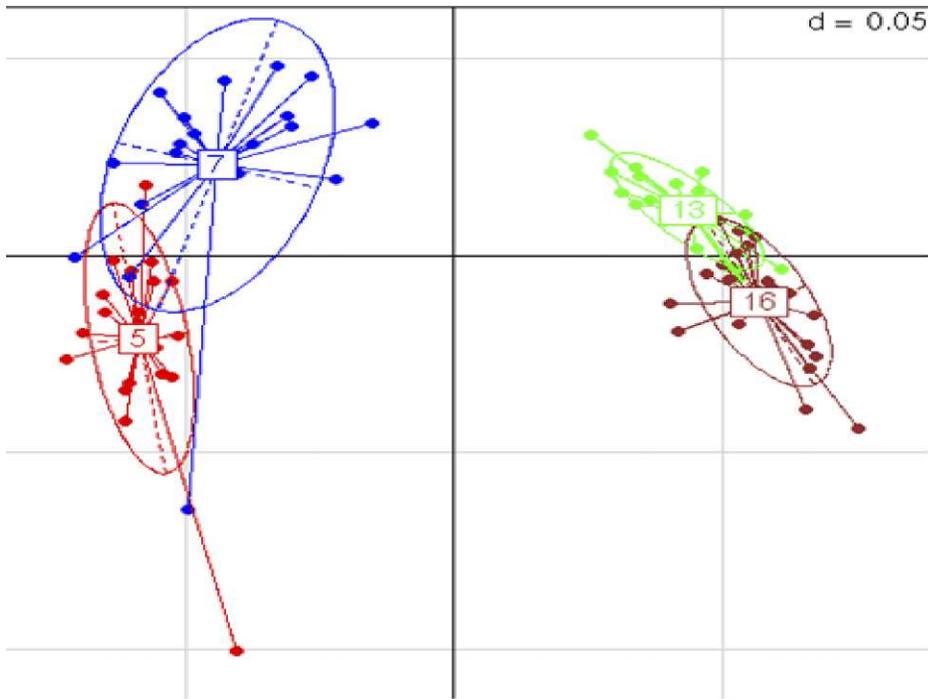
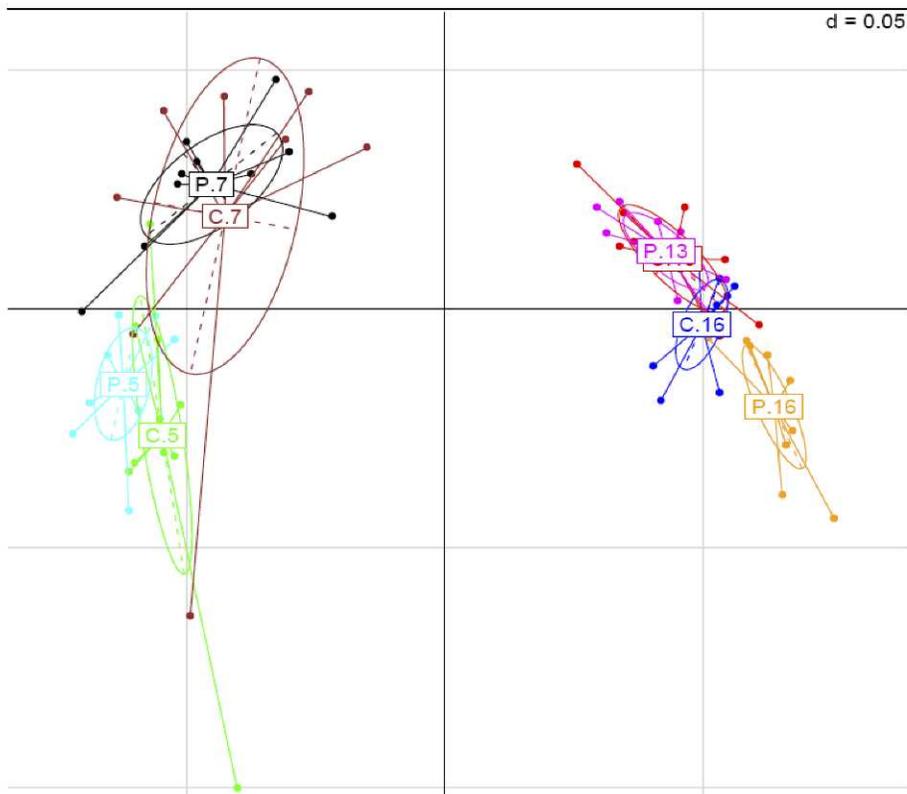


FIG. 1. Two-dimensional correspondence analysis (CoA). The CoA is a plot distribution indicating the source of greatest variation in the overall transcriptional profiles of the 80 endometrial samples with samples collected on Days 5 and 7 of the estrous cycle/early pregnancy clustered together and samples collected on Days 13 and 16 clustered together (A). Note the clear segregation between pregnant and cyclic heifers on Day 16 (B).

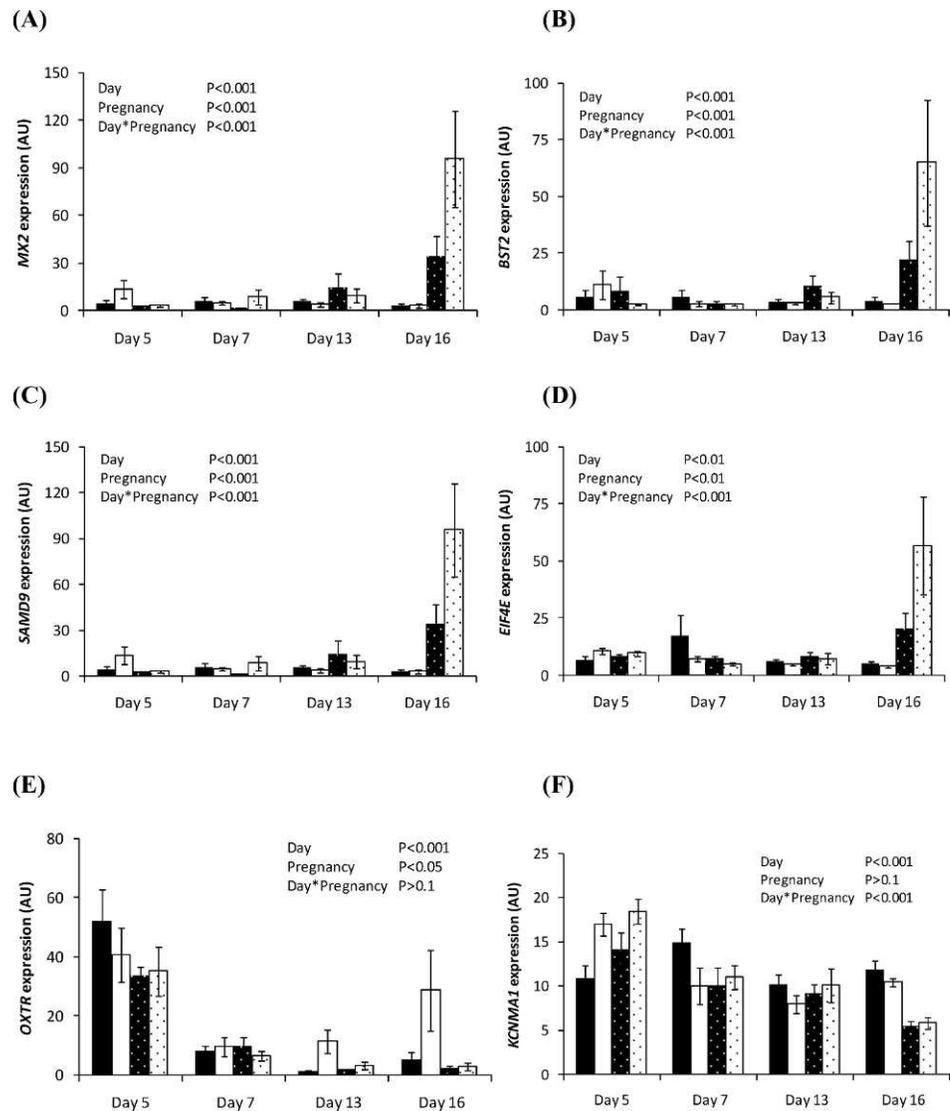
(B)



expression of 9 of those genes (*BST2*, *EIF4E*, *ISG20*, *MX2*, *OAS1*, *OXTR*, *RSAD2*, *SAMD9*, and *USP18*), with a significant pregnancy by day interaction for the expression of *BST2*, *Bt643*, *EIF4E*, *ISG20*, *KCNMA1*, *MX2*, *OAS1*, *RSAD2*,

SAMD9, and *USP18* ($P < 0.05$). There was no significant effect of progesterone on the expression of any of the genes studied. There was no treatment effect on the expression of *IFIT2* on any given day ($P > 0.1$).

FIG. 2. Real-time Q-PCR analysis of selected genes for microarray validation. Relative expression values are given in arbitrary units (AU; average \pm SEM) for (A) *MX2*, (B) *BST2*, (C) *SAMD9*, (D) *EIF4E*, (E) *OXTR*, and (F) *KCNMA1*. Cyclic, normal P4 (solid bars); cyclic, high P4 (open bars); pregnant, normal P4 (black bars white stipple) and pregnant, high P4 (white bars black stipple). Significant differences for overall effects of day, pregnancy, and Day X pregnancy interaction were set at a *P* value of <0.05 .



Gene Ontology and Ingenuity Pathway Analysis of DEGs

Analysis of the GO terms for biological processes associated with the list of 764 DEGs revealed 25 biological process terms that were overrepresented between pregnant and cyclic heifers on Day 16. Of these overrepresented GO terms only, the terms for immune response (12 genes), protein amino acid ADP-ribosylation (4 genes), response to virus (4 genes), response to biotic stimulus (5 genes), aromatic compound metabolic process (5 genes), multicellular organismal process (19 genes), heterocycle metabolic process (3 genes), transcription initiation (2 genes), and inorganic anion transport (4 genes) had two or more DEGs in their categories (see Supplemental Table S3).

IPA software identified 10 canonical pathways (Supplemental Table S4) containing genes that were significantly altered due to the presence of the conceptus at Day 16. Specific genes involved in the IFN signaling pathway (8 genes), activation of IRF cytosolic pattern recognition receptors (11 genes), retinoic acid-mediated apoptosis signaling (5 genes), role of pattern recognition receptors in recognition of bacteria (8 genes), and viruses and antigen presentation pathway (5 genes) were significantly up-regulated in pregnant endometria. Genes involved in the pathways of notch signaling, synaptic long-term depression, corticotrophin releasing hormone sig-

naling, WNT/ β -catenin signaling, and CREB signaling in neurons each contained three genes that were significantly down-regulated in pregnant compared to cyclic endometria on Day 16.

IPA interaction analysis revealed a number of interaction networks associated with the 764 DEGs detectable on Day 16. The first interaction network contained genes involved in molecular transport, genetic disorder, and immunological disease (Fig. 3) and identified an indirect interaction between the down-regulation of the *OXTR* and the signaling intermediary *MAPK3* (*ERK1*) genes. The *MAPK3* gene also indirectly interacted with IFN-alpha signaling (a type I IFN signaling mechanism), which induced the increased expression of known IFN-stimulated genes (ISGs) (e.g., *BST2* and *IFITM3*) as well as indirect interactions with the immunoproteasome and MHC class 1 complex (*PML*, *PSMB9*, *PSMB8*, *PSME2*, *PSMF1*, *PSMA2*, *TAP1*, *TAP2*). The second interaction network involved cell death, cell morphology, cellular assembly, and organization (Fig. 4) and revealed that a large number of genes involved in apoptosis were up-regulated in pregnant endometria, with *FADD*, *CASP3*, and *BCL2L1* serving as the main intermediaries for the interactions in this network. In the third interaction network, genes involved in cell death, cancer, and infection mechanisms were identified (Fig. 5). Although

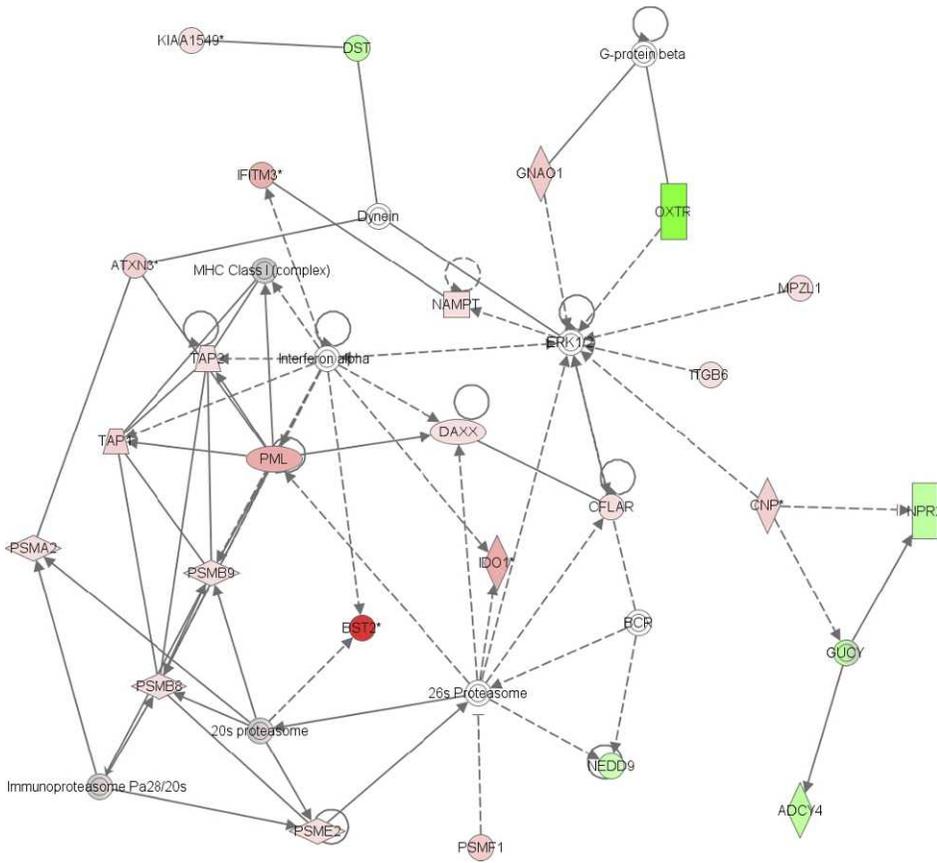


FIG. 3. Ingenuity pathway interaction network analysis 1. Differentially expressed genes on Day 16 of pregnancy/estrous cycle involved in molecular transport, genetic disorders, and immunological diseases. The network displays nodes (genes/gene products) and edges (the biological relationship between nodes). The color intensity of the nodes indicates the fold-change increase (red) or decrease (green) associated with a particular gene in endometria from pregnant compared with cyclic heifers on Day 16. A solid line indicates a direct interaction between nodes (genes/gene products) and a dashed line indicates an indirect relationship between nodes. The shape of the node is indicative of its function (see Supplemental Legend, available online www.biolreprod.org).

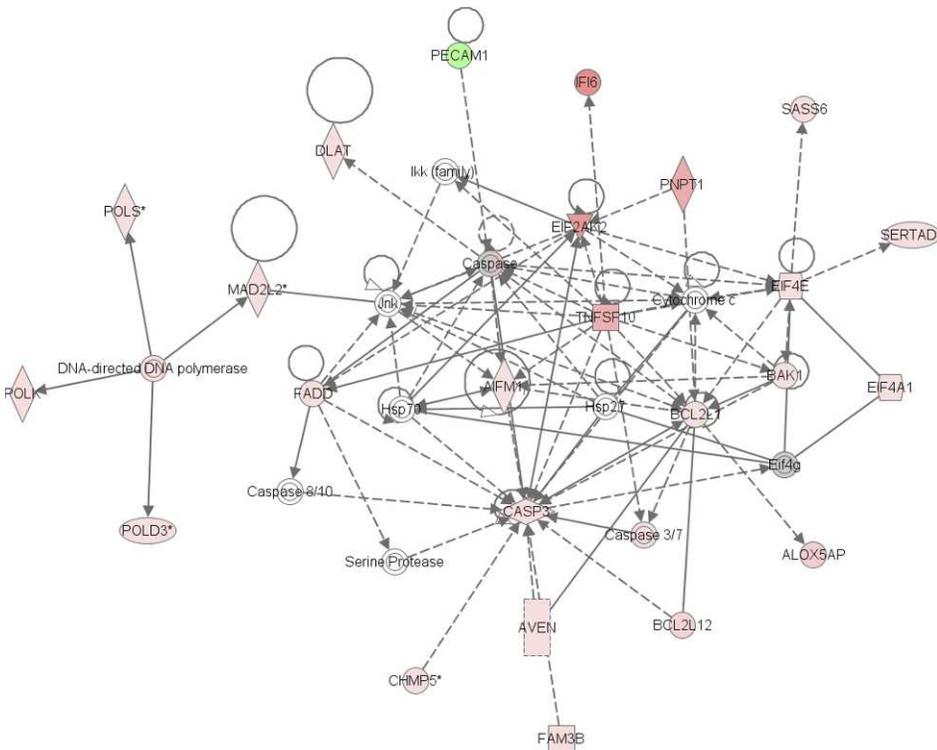
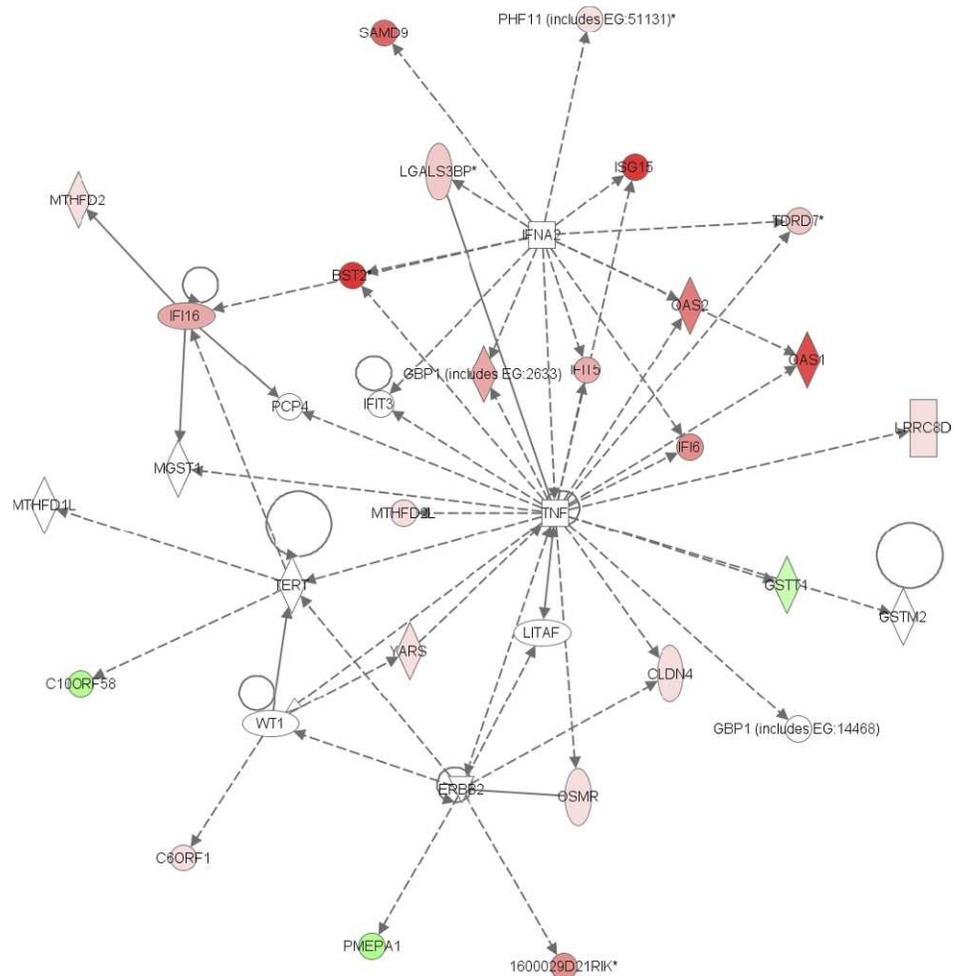


FIG. 4. Ingenuity pathway interaction network analysis 2. Differentially expressed genes on Day 16 of pregnancy/estrous cycle involved in cell death, cell morphology, cellular assembly, and organization (see legend to Fig. 3 for further explanation).

FIG. 5. Ingenuity pathway interaction network analysis 3. Differentially expressed genes on Day 16 of pregnancy/estrous cycle involved in cell death, cancer, and mechanisms for infection (see legend to Fig. 3 for further explanation).



neither *TNF* nor *IFNA2* were differentially expressed on Day 16, these molecules served as indirect links to a large number of ISGs including *ISG15*, *BST2*, *OAS2*, *OAS1*, *IFI16*, *IFI15*, *IFI6*, *LGALS3BP* (galectin 3 binding protein), and *CLDN4* (claudin 4), which are up-regulated by IFNT from the elongating conceptus in Day 16 pregnant heifers.

Exposure of Endometrial Cells to IFNT In Vitro Revealed That a Large Number of Genes Were Indirectly Regulated by IFNT in Fibroblast Cells

To determine if this panel of 12 genes was directly induced or indirectly regulated by IFNT, endometrial fibroblasts and GE cells were treated with recombinant ovine IFNT in vitro. If expression increased within 2 h of IFNT exposure, it was concluded that the gene was directly induced by IFNT. If a response was only seen following treatment with IFNT for 24 h, it was concluded that the gene was indirectly regulated by IFNT (Fig. 6). The expression of *IFIT2*, *SAMD9*, and *USP18* was directly induced by IFNT in vitro in both fibroblasts and GE cells ($P < 0.05$; Table 1). In contrast, *BST2*, *EIF4E*, *IFI144*, *ISG20*, and *MX2* expression was indirectly regulated by IFNT in fibroblasts, and only *ISG20* expression was regulated by IFNT in GE cells ($P < 0.05$). There was no effect of IFNT on expression of *Bt643*, *OXTR*, or *KCNMA1* expression in either of these uterine cell types ($P > 0.05$) (Table 1).

Infusion of IFNT In Vivo Directly Affected the Expression of Genes in the Endometrium, Predominantly in the Caruncular Regions

Infusion of the uterus in vivo with recombinant ovine IFNT revealed distinct differences in gene expression compared to the regulation of expression of these genes in cells cultured in vitro (Table 1). The expression of *BST2*, *EIF4E*, *IFIT2*, *IFI144*, *ISG20*, *MX2*, *RSAD2*, *SAMD9*, and *USP18* increased in the caruncular region of the endometrium following exposure to IFNT for 2 h compared to that in controls (Fig. 7A; $P < 0.05$). Infusion of IFNT did not significantly alter the expression of *Bt643*, *KCNMA1*, or *OXTR* ($P > 0.05$). Only *BST2*, *EIF4E*, *IFI144*, *RSAD2*, *SAMD9*, and *USP18* expression increased in the intercaruncular regions of the endometrium exposed to IFNT compared to that in controls (Fig. 7B; $P < 0.05$).

DISCUSSION

The main findings of this study are 1) under the conditions of this study, DEGs were only detectable between cyclic and pregnant endometria on Day 16 of pregnancy, coinciding with maternal recognition of pregnancy; 2) based on the rapidity of change in expression levels in vitro, some genes can be categorized as directly induced by IFNT (*IFIT2*, *SAMD9*, and *USP18*), while others are indirectly regulated by IFNT (*BST2*, *EIF4E*, *IFI144*, *ISG20*, and *MX2*); 3) in vivo analysis confirmed that IFNT directly induces the expression of *BST2*, *EIF4E*,

IFNT-Regulated genes

IFNT-Induced genes

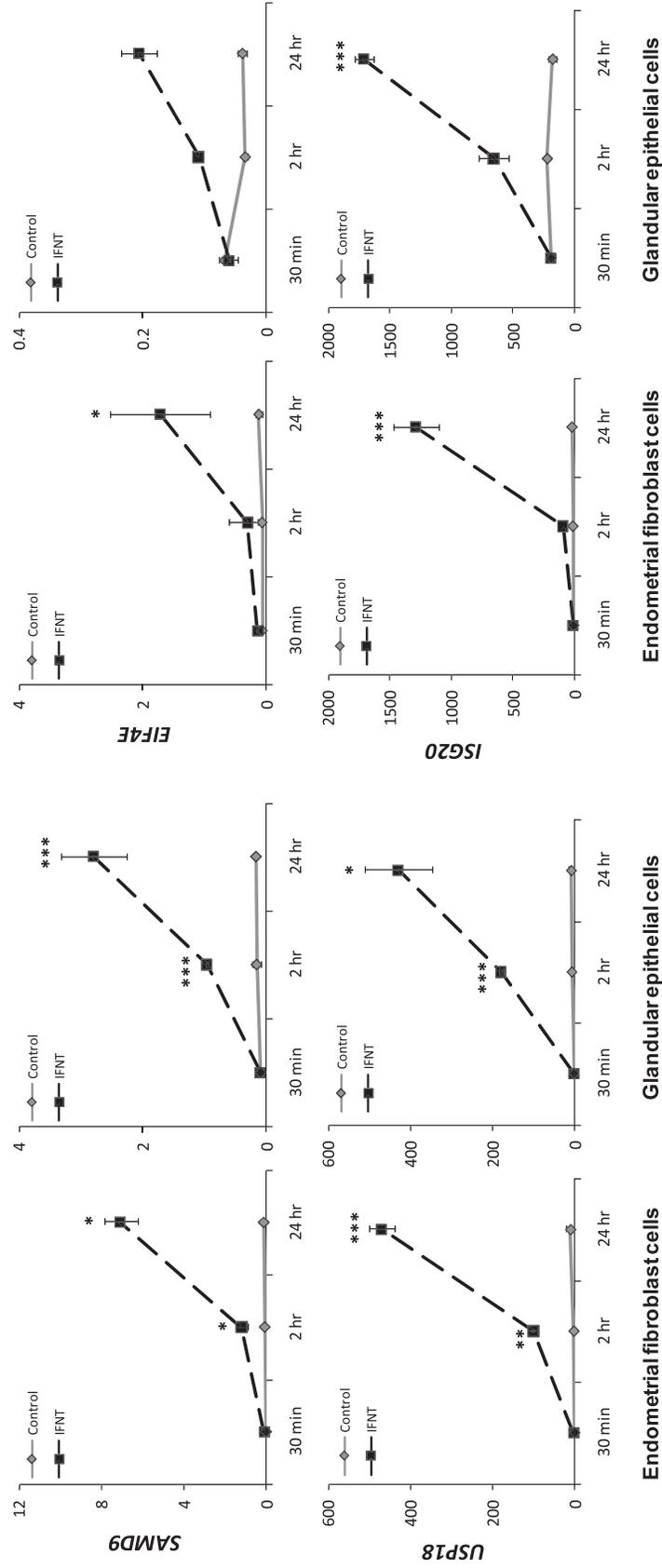


FIG. 6. Representative genes identified as either directly induced by IFNT (left panel) or indirectly regulated by IFNT (right panel) in either endometrial fibroblasts or glandular epithelial cells treated with IFNT for 30 min, 2 h, or 24 h (dashed line) compared with untreated control cells (solid line). Asterisks indicate significant effect (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) of IFNT on expression of a given gene at that time point. If the expression of a gene was significantly increased by IFNT by 20 min or 2 h after treatment, it was designated as directly induced by IFNT. If an increase in expression was only seen after 24 h, then it was concluded that the gene was indirectly regulated by IFNT.

TABLE 1. Q-RT-PCR analysis summary of genes altered to the greatest extent due to the presence of a conceptus on Day 16 of pregnancy in 1) endometria of pregnant and noninseminated heifers; 2) endometrial cells cultured in vitro with or without interferon tau (IFNT) for 30 min, 2 h, or 24 h; and 3) endometria recovered from heifers exposed to IFNT in vivo for 2 h.^a

Gene	In vivo pregnant vs. cyclic			In vitro IFNT ^b						In vivo IFNT ^c	
	Endometrial homogenate			Fibroblast cells			Glandular epithelial cells			CAR	ICAR
	Day	Pregnancy	Day × Pregnancy	30 min	2 h	24 h	30 min	2 h	24 h	2 h	2 h
<i>BST2</i>	***	***	***	—	—	***	—	—	—	**	*
<i>Bt643</i>	***	—	*	—	—	—	—	—	—	—	—
<i>EIF4E</i>	**	**	***	—	—	*	—	—	—	***	*
<i>IFIT2</i>	—	—	—	—	*	***	—	*	—	*	—
<i>IFI44</i>	*	*	**	—	—	**	—	—	—	***	—
<i>ISG20</i>	***	—	***	—	—	***	—	—	***	***	—
<i>KCNMA1</i>	**	**	***	—	—	—	—	—	—	—	—
<i>MX2</i>	***	***	***	—	—	**	—	—	—	*	—
<i>OXTR</i>	***	*	—	—	—	—	—	—	—	—	—
<i>RSAD2^d</i>	*	**	**	—	*	***	—	**	*	***	*
<i>SAMD9</i>	***	***	***	—	*	*	—	***	***	**	*
<i>USP18</i>	***	***	***	—	**	***	—	***	*	***	**

^a A significant effect of treatment is indicated by an asterisk: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

^b Genes regulated at 30 min or 2 h were considered as early interferon (IFNT)-induced genes, and genes regulated at 24 h were considered as IFNT-regulated genes. The regulation in each endometrial cell type tested is indicated.

^c CAR, caruncular region; ICAR, inter-caruncular regions.

^d In vitro IFNT data previously published [16].

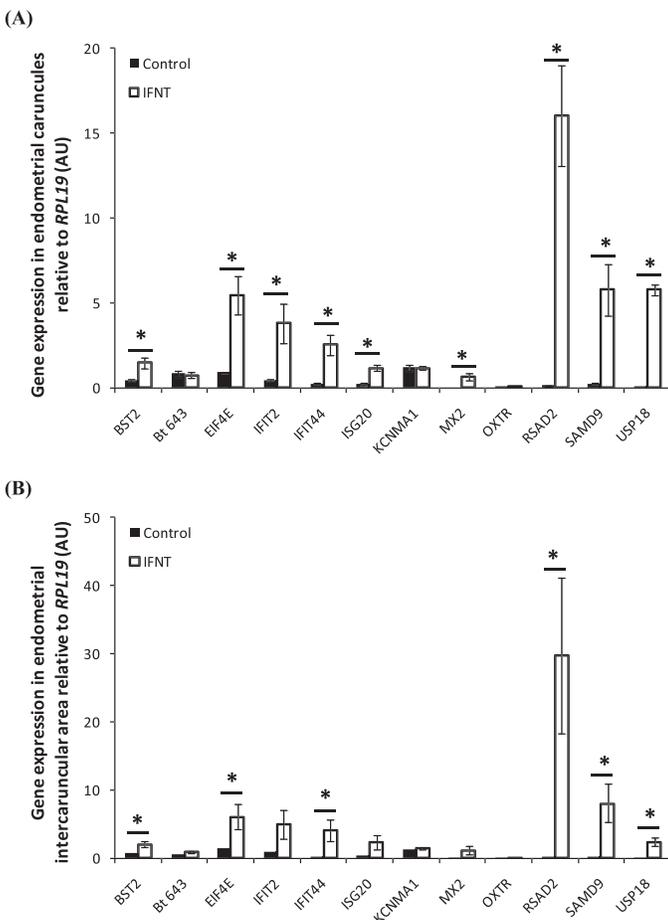


FIG. 7. Gene expression relative to *RPL19* (normalizer gene) in arbitrary units (AU) in the endometria of control heifers (solid bars) or heifers treated with IFNT for 2 h (open bars) in the caruncular (A) and intercaruncular (B) regions of the uterine endometrium. An asterisk (*) denotes a significant ($P < 0.05$) increase in gene expression in IFNT-treated compared to control endometria.

IFIT2, *IFI44*, *ISG20*, *MX2*, *RSAD2*, *SAMD9*, and *USP18*; and 4) short-term treatment with IFNT did not effect the expression of *OXTR*, *Bt643*, or *KCNMA1* either in vitro or in vivo. This study is the first to identify the key stage in early pregnancy when the endometrium becomes responsive to signaling from the developing conceptus. The results of this study clearly indicate that it is only at maternal recognition of pregnancy (Day 16) that differences in the endometrial transcriptome are first detectable between pregnant and cyclic heifers. This is consistent with the fact that synchronous transfer of embryos in cattle on Day 16 but not Day 17 extends the luteal life span [10] as well as infusion of embryonic homogenates on Days 17, 18, and 19 [9] and is coordinate with increased IFNT production by the conceptus trophoblast [28–30]. Thus, the transcriptional changes that occur in the endometrium throughout the estrous cycle take place irrespective of whether a conceptus is present, and a divergence in overall endometrial gene expression induced by the conceptus is only apparent during pregnancy recognition. Indeed, throughout the luteal phase of the estrous cycle and early pregnancy, both the pregnant and cyclic groups of heifers are exposed to the same hormonal environment. Changes that do occur in endometrial gene expression are governed by follicular estrogen and luteal progesterone, the actions of which are mediated through their receptors in cells of the uterus. It is only at pregnancy recognition, when conceptus-derived IFNT prevents luteolysis, that the hormonal environment, and thus the endocrine mechanisms regulating endometrial gene expression, differs between pregnant and cyclic heifers. Moreover, the lack of difference in endometrial gene expression at Days 5, 7, and 13 is consistent with the fact that it is possible to transfer a Day-7 blastocyst into the uterus of synchronized heifers on Day 7 of the estrous cycle and achieve an acceptable pregnancy rate. This suggests that the embryo is relatively autonomous for at least the first week of life and that direct contact with the maternal reproductive tract is not strictly necessary to develop to the blastocyst stage. Further evidence of this is provided by the ability to grow blastocysts in vitro in the absence of any direct contact with the female reproductive tract in large

numbers using in vitro maturation/in vitro fertilization technology.

Thus, up to at least Day 13, the uterus of the cow is “optimistic,” expecting and preparing for pregnancy. Evidence for this receptivity is seen in the fact that increasing circulating concentrations of progesterone down-regulate the expression of the progesterone receptor (PGR) from the LE and GE to establish uterine receptivity to implantation, irrespective of whether the animal is pregnant or not, in all mammalian species studied [31–34]. Endometrial stromal cells maintain expression of PGR and produce growth factors (e.g., fibroblast growth factor 7 [FGF7], and FGF10; hepatocyte growth factor [HGF]; and insulin-like growth factor 1 [IGF1] and IGF2; [35–39]) of which FGF10 is maintained by progesterone. Interestingly, these stromally derived growth factors have similar expression patterns in both pregnant and cyclic ewes prior to maternal recognition of pregnancy [27–31]. The present study found no differences in the expression of these genes prior to Day 16 in cattle, indicating that the mechanisms to establish uterine receptivity and to promote conceptus elongation are in place in the endometrium of both cyclic ewes and heifers prior to pregnancy recognition.

Previous studies in cattle have identified the fact that the endometrium can be used as a sensor for the type of conceptus present (in vivo, in vitro, or clone-derived conceptus [20, 21]); however, this response was only examined at the late peri-implantation stages of pregnancy (Days 18 and 20). It is known that conceptus trophoblast expresses mRNA for IFNT as early as the blastocyst stage of development and can secrete detectable amounts when cultured in vitro [40, 41]. While it is possible that this production of IFNT early in development may elicit a local effect on gene expression in the endometrium, this may occur in a cell-specific manner that was not detectable in the present study. Moreover, in the present model, insertion of a progesterone-releasing intravaginal device did increase conceptus size on Day 13 and Day 16 [23], and the more advanced Day 13 conceptus may also elicit a local response in the endometrium that may not have been of a sufficient magnitude to be detectable under the conditions of this study.

All available analyses, including GO and IPA, determined that the DEGs identified on Day 16 of pregnancy are due to the presence of the conceptus and that the majority were expressed in response to IFNT produced by the conceptus. IFNT is a type I interferon that has antiviral, antiproliferative, and immunosuppressive roles. IFNT mediates its actions in the endometrium in two ways. Following binding of IFNT to its receptors (IFNAR1 and IFNAR2), it initiates cell signaling via the Janus activation kinases (JAKs) and tyrosine kinase 2 (TYK2) [42]. IFNT then induces the expression of classical ISGs in the stromal cells that express interferon sensitive response elements or ISREs. In contrast, expression of interferon regulatory factor 2 (IRF2) in the LE and GE of sheep inhibits classical ISG expression [43]. However, IFNT does stimulate a number of genes in the endometrial epithelia that transport nutrients or enhance genes for proteins important for conceptus elongation and uterine receptivity to implantation [42]. In sheep, the expression of these genes occurs in both cyclic and pregnant animals and is initially induced by progesterone and then further stimulated by trophoblast-produced IFNT (galectin 15 or *LGALS15* [44]; gastrin releasing peptide, *GRP* [45]; hypoxia inducing factor 1A, *HIF1A* [46]; cystatin C, *CST3* [47]; cathepsin L, *CTSL* [48]; insulin-like growth factor binding protein 1, *IGFBP1* [49]; leukemia inhibitory factor receptor, *LIFR*, and interleukin 6 signal transducer, *IL6ST* [50]; members of the solute carrier family of transporters [51–54]; and selected nutrients [55, 56]) as well as connective tissue

growth factor (*CTGF*) and solute carrier family member 5A1 (*SLC5A1*) in cattle [57]. This, in conjunction with the present results, indicates that the transcriptional changes that take place in the endometrium prior to maternal recognition of pregnancy occur irrespective of whether a conceptus is present. The results from the present experiment indicate that some of these genes (involved in endometrial remodeling for implantation) are stimulated in pregnant heifers on Day 16 as increased expression of the adhesion factors *LGALS3BP* and *LGALS9*, and the matrix-associated factors *CLDN4* [58] are detectable. This suggests that the mechanisms required for adhesion and apposition of the elongating conceptus to uterine LE during the peri-implantation period are further enhanced by IFNT secretion on Day 16. This is further strengthened by the interaction network analysis (Fig. 5) that provides indirect links between interferon receptor alpha 2 (IFNA2) and a number of these genes.

Of the DEGs identified on Day 16 of pregnancy, several are classical ISGs involved in type I interferon signaling in both cattle and sheep [59], and others were associated with the overrepresented GO terms Immune Response and ISG15-protein conjugation, as well as the pathways of interferon signaling and activation of IRF cytosolic pattern recognition receptors. This indicates that the earliest detectable differences in endometrial gene expression between pregnant and cyclic heifers are a response of the endometrium to IFNT secreted (and possibly other conceptus-derived factors) by conceptus trophoblast (e.g., *B2M* [60], *MX1* [61], *IL15*, *ISG15* [62, 63], *OAS1* [64, 65], *LOC512486*, *MGC151529*, *GBP5*, *GBP4*, *CXCL10*, *LOC781225*, and *C4A*). Other studies in cattle revealed that both the classical and “novel” ISGs are differentially expressed on Day 18 (109 DEGs) and Day 20 (1295 DEGs) of pregnancy using different microarray platforms [21, 66]. Of the DEGs identified at the peri-implantation period of pregnancy, 27 are differentially expressed on Day 16 as part of the early endometrial response to the conceptus (*ACO2*, *B2M*, *BIRC4BP*, *C4A*, *C14orf10*, *CD81*, *CIR*, *CLDN4*, *EIF4E*, *EPST11*, *IFI6*, *GABARAPL1*, *ISG12(A)*, *ISG15*, *ISGF3G*, *LGP2*, *LGALS9*, *LGALS3BP*, *MX2*, *MX1*, *OAS1*, *PSMF1*, *RTP4*, *STAT1*, *SHISA5* [*SCOTIN*], *UBE1L*, and *WARS*) and represent early endometrial markers of a viable preimplantation conceptus.

In addition to inducing the expression of genes that control cell proliferation (*SAMD9*, *EIF4E*), IFNT also effects the expression of apoptotic genes in vitro [67]. The second interaction network identified by IPA (Fig. 4) revealed that a large number of genes involved in apoptosis were up-regulated in pregnant endometria on Day 16 with the genes *FADD*, *CASP3*, and *BCL2L1* serving as the main intermediaries for this interaction network. However, both proapoptotic (*TNFSF10*, *FADD*, *BAK1*, *CASP3/7*) and antiapoptotic (*BCL2L1*, *BCL2L12*) genes are up-regulated during pregnancy recognition. Recent reports have shown that Fas ligand (*FASLG*) is secreted by the conceptus, and, although this trigger of apoptosis is produced by the conceptus and the expression of proapoptotic genes is increased in bovine endometrium at the time of trophoblast attachment, there is no additional increase in apoptosis in the uterine endometrium [68]. Our results suggest that while conceptus-derived factors enhance the expression of a number of proapoptotic genes, antiapoptotic genes are also up-regulated, indicating that a mechanism to balance the apoptotic process required in the maternal endometrium for implantation to occur is in place during pregnancy recognition.

To determine how the expression panel of DEGs was regulated in pregnant heifers, we examined their expression in

endometrial fibroblasts and glandular epithelial cells treated with or without IFNT in vitro. Interestingly, the expression of only three genes was induced directly by IFNT (*IFIT2*, *SAMD9*, and *USP18*) with a further five genes regulated indirectly by IFNT (*BST2*, *EIF4E*, *IFI44*, *ISG20*, and *MX2*). Given that *MX2* and *BST2* were previously identified as directly induced in cattle in vitro by IFNT [18] and in sheep both in vitro and in vivo by IFNT [69], these results were surprising. To ensure that this was not an artifact of the in vitro system, further validation on the IFNT regulation of these genes was carried out in vivo. In this model, all nine genes that increased in pregnant heifers to the greatest extent on Day 16 were directly induced by IFNT, i.e., following IFNT infusion for 2 h in the uteri of cyclic heifers. These results confirmed that these genes elicit the largest detectable endometrial response to the presence of the conceptus at maternal recognition of pregnancy.

Results of this study also provide direct evidence that both *SAMD9* and *EIF4E* are novel early response genes in the bovine endometrium to the conceptus, as IFNT directly induced expression of these genes both in vivo and in vitro. The *SAMD9* gene, first identified as a deletion in humans causing normophosphatemic familial tumoral calcinosis [70], is a potent tumor suppressor [71] and its expression is down-regulated in aggressive fibromatosis in humans [72]. Overexpression of *SAMD9* negatively regulates tumor cell growth and invasion and knockdown of *SAMD9* inhibits apoptosis. In addition, *SAMD9* reduces cell proliferation and is required to inhibit IFN-induced cell proliferation and migration of activated CD4⁺ T cells [73]. Given that *SAMD9* inhibits cell proliferation and migration, its induction by IFNT may play a role in preventing excessive conceptus trophoblast invasion into the stromal cells during early pregnancy in cattle. *EIF4E*, by complexing with *EIF4A* and *4G* [74], stimulates translation of capped mRNAs [75, 76] that are involved in cell proliferation, growth, and apoptosis. A truncated form of this protein has been identified at sites of implantation in pigs, and it is induced in the pig in a time of high P4 and likely by the pregnancy recognition signal, estradiol [77]. Here we provide evidence that the expression of *EIF4E* is directly induced by IFNT in both caruncular and intercaruncular regions of the bovine endometrium during pregnancy recognition, and it may play a role in maintaining cell proliferation at this time.

Despite the fact that IFNT directly induces the expression of the top up-regulated genes at maternal recognition of pregnancy, neither in vitro nor in vivo IFNT treatment altered the expression of *OXTR*, *Bt.643*, and *KCNMA1*, genes that were decreased to the greatest extent due to pregnancy. Given that the uterus was only exposed to IFNT for 2 h in vivo, i.e., short-term exposure, this may explain why there was no effect on the expression of these genes. The expression of the *OXTR* decreased to the greatest extent in pregnant endometria, and the proposed mechanism through which CL regression is inhibited is via IFNT inhibition of estrogen receptor alpha expression (*ESR1*). Estrogen, acting via *ESR1*, drives transcription of the oxytocin receptor (*OXTR*), and without the expression of the *OXTR*, oxytocin cannot induce secretion of oxytocin-induced luteolytic pulses of PG by the uterus [59, 78]. Interestingly, the presence of a conceptus had no effect on the expression of the *ESR1* gene and confirms previous findings by our group and others that neither *ESR1* gene expression nor protein localization is affected by the presence of a conceptus on Day 16 [33, 79, 80]. Intrauterine infusion of both recombinant bovine and ovine IFNT prolongs luteal life span and prevents oxytocin-induced increase of endometrial PG in dairy cows [81]; therefore, some additional mechanism and not *ESR1*-

driven *OXTR* expression must play a role in preventing luteolysis in pregnant and IFNT-infused uteri. Recent studies by Bott et al. [82] have shown that IFNT can act in an endocrine manner and extend the life span of the CL. Moreover, when IFNT was infused into the uterine vein and PG was administered, P4 output, although it initially declined, recovered within 8 h compared to that of controls. These data as well as results presented in this study suggest that it may be the endocrine actions of IFNT that prevent luteolysis of the CL in the bovine. What is clear from this study is that by Day 16 in cyclic heifers, the luteolytic mechanism that was abrogated in pregnant heifers has already developed and is detectable in the endometria of cyclic heifers [80].

In summary, successful establishment of pregnancy in ruminants requires a sequence of key events to ensure uterine receptivity to implantation, including progesterone-induced alterations in endometrial gene expression [8, 24, 83, 84] as well as down-regulation of its own nuclear receptor in uterine LE and GE [31–33]. In addition, an adequate endometrial response to IFNT production is required, including further stimulation of expression of P4-induced genes in the LE and GE, induction of ISGs in the endometrial stroma. This study is the first to show that changes that occur in the endometrial transcriptome are independent of the presence of a conceptus until maternal recognition of pregnancy and that the requirements for uterine receptivity are established prior to pregnancy recognition in both pregnant and cyclic heifers. The DEGs increased by Day 16 are largely in response to IFNT production, and the genes increased to the greatest extent are directly induced by IFNT in vivo. These genes represent known and novel early markers of successful preimplantation conceptus development and/or return to cyclicity and identify the earliest stage at which an endometrial response to the conceptus is detectable.

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