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Progesterone-Regulated Changes in Endometrial Gene Expression Contribute to Advanced Conceptus Development in Cattle[1]

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ABSTRACT

The postovulatory rise in circulating progesterone (P4) concentrations is associated with increased pregnancy success in beef and dairy cattle. Our study objective was to determine how elevated P4 alters endometrial gene expression to advance conceptus development. Synchronized heifers were inseminated (Day 0) and randomly assigned to pregnant high P4 or to pregnant normal P4. All high P4 groups received a P4-release intravaginal device on Day 3 after insemination that increased P4 concentrations up to Day 7 ($P < 0.05$). Tissue was collected on Day 5, 7, 13, or 16 of pregnancy, and endometrial gene expression was analyzed using the bovine Affymetrix (Santa Clara, CA) microarrays. Microarray analyses demonstrated that the largest number of P4-regulated genes coincided with the day when the P4 profiles were different for the longest period. Genes with the largest fold change increase (such as *DGAT2* and *MSTN* [also known as *GDF8*]) were associated with triglyceride synthesis and glucose transport, which can be utilized as an energy source for the developing embryo. Temporal changes occurred at different stages of early pregnancy, with the greatest difference occurring between well-separated stages of conceptus development. Validation of a number of genes by quantitative real-time PCR indicated that P4 supplementation advances endometrial gene expression by altering the time (*FABP*, *DGAT2*, and *MSTN*) or duration (*CRYGS*) of expression pattern for genes that contribute to the composition of histotroph.

female reproductive tract, gene regulation, pregnancy, progesterone, uterus

INTRODUCTION

Embryonic and fetal mortality rates are about 40% in cattle based on a fertilization rate of 90% and an average calving rate of about 55%; the majority of this loss (70%–80%) occurs between Day 8 and Day 16 after insemination [1–3]. During this critical window, the elongating conceptus (embryo and associated extraembryonic membranes) must secrete sufficient quantities of interferon tau (IFNT) to signal pregnancy recognition and to prevent uterine release of luteolytic pulses of prostaglandin F2 alpha [4, 5].

The steroid hormone progesterone (P4) has a key role in the establishment and maintenance of pregnancy. Retrospective studies [1, 6–8] in both beef heifers and dairy cows detected positive correlations between naturally occurring elevated concentrations of P4 in the immediate postconception period, which rise or increase up to Day 7, and the likelihood of conceptus survival and establishment of pregnancy. Indeed, delivery of exogenous P4 to increase circulating concentrations in the first few days after conception advances posthatching expansion and elongation of conceptuses in both cattle [9, 10] and sheep [11] and is associated with earlier onset of IFNT secretion [12].

The mechanism of action whereby P4 stimulates conceptus development has not been elucidated, but available evidence indicates that P4-induced changes in endometrial gene expression lead to changes in the composition of histotroph that are required for posthatching conceptus survival and growth [13]. The importance of histotroph for conceptus development was demonstrated in the uterine gland knockout (UGKO) model in sheep in which embryos fail to develop beyond the blastocyst stage (i.e., Day 14) in adult UGKO ewes [14]. It is likely that P4 affects conceptus development, at least in part, by altering the temporal and spatial expression of key regulatory genes required as part of the biochemical cascade that controls early embryonic development by preparing the endometrium for implantation [13].

To date, transcriptional profiling of the female reproductive tract in cattle has focused on expression profiles of bovine epithelial cells of the ipsilateral (same side as the ovary containing the corpus luteum) vs. the contralateral oviduct [15], as well as comparisons among cells in the ipsilateral oviduct [16] or uterine endometrium [17]. Another study [18] examined differences in endometrial gene expression at different stages of the estrous cycle, with specific emphasis on estrus (low P4) and diestrus (high P4). Molecular pathways modified by different P4 environments were identified, but these studies [17, 18] were limited to nonpregnant cattle. Despite a significant body of evidence indicating that circulating concentrations of P4 in the immediate postconception period are closely associated with conceptus development and pregnancy success, there has been no comprehensive study to date of the transcriptional profile of the endometrium at different stages of early pregnancy in cattle and the effect of P4.

We hypothesized that increasing concentrations of circulating P4 by exogenous supplementation would alter the pattern of endometrial gene expression and composition of histotroph to create an environment most likely to promote expansion of

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blastocysts and elongation of conceptuses during the periimplantation period of pregnancy. Therefore, the objectives of this study were 1) to determine how endometrial gene expression in early pregnant heifers is altered by elevated P4, 2) to describe the temporal changes in gene expression occurring during early pregnancy in animals with normal P4 and high P4, and 3) to ascertain P4-regulated genes in the endometrium that likely regulate conceptus elongation.

MATERIALS AND METHODS

Animals and Treatments

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland. Protocols were in accord with the Cruelty to Animals Act (Ireland 1897) and the European Community Directive 86/609/EC and were sanctioned by the University College Dublin Animal Research Ethics Committee.

This study was carried out as part of a larger study examining changes in gene expression in endometria of cyclic and pregnant heifers; however, only data relating to P4-induced changes in endometrial gene expression in pregnant heifers are reported herein. The experimental design has been described previously [10]. Briefly, estrus in crossbred beef heifers (n = 263) was synchronized using a controlled internal drug-release device (CIDR; InterAg, Hamilton, New Zealand), which remained in situ in the uterus for 8 days. Three days before CIDR removal, heifers received 15 mg of prostaglandin $F_{2\alpha}$ (Estrumate; Shering-Plough Animal Health, Hertfordshire, UK). Sixty hours after CIDR removal, heifers were checked for standing estrus, and only those in estrus (Day 0) were used in the studies.

To ensure a sufficient number of pregnant heifers, 139 heifers were artificially inseminated with semen from a proven sire. Approximately one half of the heifers received a P4-releasing intravaginal device (PRID) containing 1.55 g of P4 (CEVA Animal Health Ltd., Chesham, UK) on Day 3 of the estrous cycle to increase circulating concentrations of P4. PRIDs were not inserted earlier than this to avoid potential negative effects on fertility [16]. Thus, the two treatment groups were 1) pregnant high P4 (PH [n = 73]) and 2) pregnant normal P4 (PN [n = 66]) (Fig. 1). Blood samples were taken from all heifers twice daily from Day 0 to Day 7 after estrus and once daily thereafter until slaughter to characterize the postovulatory changes in concentrations of P4 in serum. Heifers were slaughtered on Day 5 (n = 26), 7 (n = 37), 13 (n = 48), or 16 (n = 28), corresponding to 16-cell/early morula-stage embryos, blastocyst stage, initiation of conceptus elongation, and time of advanced conceptus elongation and secretion of IFNT for maternal recognition of pregnancy, respectively.

Tissue Collection

Approximately 30 min after slaughter, the reproductive tract of each heifer was flushed with PBS containing 5% fetal calf serum. Pregnancy was confirmed by the presence of a morphologically normal embryo/conceptus in the flush using a stereomicroscope. To remove potential ambiguity from the results, heifers that were inseminated but not pregnant at slaughter were removed from the experiment.

After flushing, the uterine horn ipsilateral to the ovary containing the corpus luteum was opened longitudinally, and strips of endometrium (caruncular and intercaruncular) were carefully removed using curved scissors. Strips of endometrium of approximately 300 mg each were immersed in 1:5 w/v of RNAlater (Sigma, Dublin, Ireland), transported on ice back to the laboratory, and stored at 4°C for 24 h. Samples were then transferred from the RNAlater to RNase/DNase-free tubes and stored at −80°C until analyzed pending the results of the P4 profiles.

Concentrations of P4 in sera were determined by time-resolved fluoroimmunoassay using an AutoDELFIA Progesterone Kit (Perkin Elmer, Wallac Oy, Turku, Finland) [10]. Heifers were selected for microarray analyses (n = 5 per group per day, for a total of 40 arrays) on the basis of their P4 profile. Heifers that received a PRID to increase P4 concentrations were chosen as those with the greatest deviation from the mean to avoid an overlap in P4 profiles between PH heifers with "low" P4 and PN heifers with higher P4 profiles.

RNA Isolation and Microarray Hybridization

Once the heifers were chosen for microarray analysis, total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. Briefly, 100 mg of endometrium was homogenized, 5 ml of Trizol was added, and chloroform and isopropanol steps were performed. Following extraction, RNA was resuspended in 100 μl of RNase/DNase-free water. RNA cleanup and on-column DNase digestion were performed using the Qiagen Mini Kit (Qiagen, Crawley, West Sussex, UK). Total RNA quality and quantity were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Experimental Design

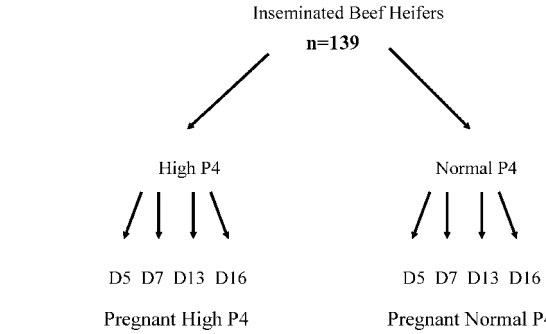

FIG. 1. Experimental design for tissue collection and microarray analyses. Only heifers with the appropriate P4 profile and a viable conceptus were used for further analysis. Heifers were assigned to slaughter on either Day (D) 5, 7, 13, or 16 of pregnancy (n = 5 per treatment per day).

All microarray protocols were carried out by Almac Diagnostics Ltd. (Craigavon, Armagh, Northern Ireland). One-cycle target labeling was performed using 2 μg of total RNA with an RNA integrity number of greater than 8.5. Total RNA was converted to cDNA via first- and second-strand synthesis using the GeneChip (Affymetrix, Santa Clara, CA) Expression 3′-Amplification One-Cycle cDNA Synthesis Kit, in conjunction with the GeneChip Eukaryotic PolyA RNA Control Kit. Double-stranded cDNA cleanup was carried out using the GeneChip Sample Cleanup Module. Biotin-labeled cRNA was synthesized from double-stranded cDNA using the GeneChip Expression 3′-Amplification In Vitro Transcription Labeling Kit. Biotin-labeled cRNA cleanup removed unincorporated nucleotide triphosphates using the GeneChip Sample Cleanup Module, and cRNA quality was assessed using an Eppendorf BioPhotometer (Eppendorf International, Westbury, NY) and the Agilent 2100 Bioanalyzer. Twenty-five micrograms of cRNA generated in the in vitro transcription reaction was fragmented using 5× fragmentation buffer and RNase-free water contained within the GeneChip Sample Cleanup Module. The fragmentation reaction was carried out at 94°C for 35 min to generate fragments of 35–200 base pair for hybridization. The quality of fragmented cRNA was assessed using the Agilent 2100 Bioanalyzer.

Before hybridization, the adjusted cRNA yield in the fragmentation reaction was calculated to account for carryover of total RNA in the in vitro transcription reaction. Fifteen micrograms of fragmented cRNA was made up in a hybridization cocktail in accord with the Affymetrix technical manual corresponding to a 49-format (standard)/64-format array. The hybridization cocktail was added to the GeneChip Bovine Genome Array and hybridized for 16 h at 45°C. The array was then washed and stained on the GeneChip Fluidics Station 450 using the appropriate fluidics script; once completed, the array was inserted into the Affymetrix AutoLoader Carousel and scanned using the GeneChip Scanner 3000.

Data Analysis

The raw data intensity files were read into R (http://www.r-project.org/) and preprocessed using functions of the Affymetrix and Gene Chip Robust Multi-array Average packages of the BioConductor project (http://www.bioconductor.org/) [19]. Identification of differentially expressed genes (DEGs) was achieved using the limma package in R/BioConductor via 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. DEGs were selected on the basis of an adjusted $P < 0.05$. For gene ontology (GO) overrepresentation analysis, a more stringent $P < 0.01$ was used as the cutoff point for inputting gene lists into the GOstats package of BioConductor (described herein).

GO Analysis

Analysis of the GO terms was performed using the GOstats package of BioConductor [20]. The chip probes were first filtered as outlined in GOstats

Vignette or the Help Page in BioConductor. This provided a "gene universe" that represented the set of expressed genes across experimental conditions. The filtering reduced the amount 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 significant probes generated from the microarray analysis, a conditional hypergeometric statistical test was performed using a $P < 0.01$ cutoff. This selected the overrepresented GO nodes (i.e., those associated with the probe list more 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. A correspondence analysis was performed to determine the greatest source of variation in the tissue samples.

Quantitative Real-Time PCR Validation

To validate the findings from the microarray procedure, quantitative real-time PCR was performed on six genes known to be differentially expressed on the microarray (Table 1). RNA, previously extracted for the microarray study, was converted to cDNA with Superscript III (Invitrogen) using 5 μg of total RNA and random hexamers per the manufacturer's instructions. Complementary DNA was quantified and diluted to 10 ng/μl. Primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was carried out on the 7500 Fast Real-Time PCR System (Applied Biosystems). Each reaction consisted of 50 ng of cDNA, primer concentrations given in Table 2, and 10 μl of SYBR Green Mastermix (Applied Biosystems), with a final reaction volume to 20 μl with RNase/DNase-free water. Cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min. A dissociation curve was included to ensure specificity of amplification.

A reference (housekeeping gene) study was performed using geNorm (http://medgen.ugent.be/~jvdesomp/genorm/) to determine the most appropriate reference gene for our model system [21]. *ACTB* was the most stable reference gene in the tissue samples. Transcript abundance was determined relative to the reference gene (*ACTB*) using the delta Ct method, and relative abundance values were compared using Student *t*-test.

RESULTS

Correspondence analysis, a computational method to study the association between variables, was used to obtain a visual understanding of the relationship between the different experimental components (Fig. 2). Using only pregnant heifers, the analysis resulted in a clear segregation of the samples into four clusters corresponding to Days 5, 7, 13, and 16, with Day 5 and Day 7 samples clustered together and Day 13 and Day 16 samples clustered together (Fig. 2A). Moreover, correspondence analysis that included the P4 status of the heifers revealed that (within each stage of pregnancy) PH heifers clustered together and PN heifers clustered together, with the clearest P4-associated differences in gene expression visible on Day 7 and Day 16 of pregnancy (Fig. 2B).

P4 Supplementation Induces Changes in Endometrial Gene Expression at Key Stages of Embryo Development

To address the question of how elevated P4 affected endometrial gene expression on specific days of pregnancy, endometria from PH heifers were compared with endometria from PN heifers on the same day. On Day 5 and Day 7, elevated P4 was associated with 36 and 124 DEGs, respectively ($P < 0.05$) (Table 2), which were overrepresented in a number of GO categories. The magnitude of change in P4-regulated genes was small, with only six genes on Day 5 (*IBSP*, *LOC782061*, *MSTN*, *DGAT2*, *LOC515128*, and *PRR5L*) and nine genes on Day 7 (*DGAT2*, *HPSE*, *SLC6A20*, *FABP*, *SVS8*, *CORO2A*, *ELL3*, *UNC93A*, and *SYT7*) exhibiting a greater than 2-fold increase or decrease in expression. On Day 13 and Day 16, there were few DEGs between PH heifers and PN heifers (15 and 25, respectively; $P < 0.05$) (Table 2) and no overrepresented GO categories (Supplemental Tables S1 and S2 available at www.biolreprod.org).

TABLE 1. Full names, abbreviations, accession numbers, and primer sequences for all genes used in geNorm study and Q-RT-PCR validation of microarray data.[a]

Accession no.	Gene symbol	Gene name	Forward primer	Reverse primer	Concentration (nmol)
AV607592	*ACTB*	Actin, beta	CGCCATGGATGATGATATTGC	AAGCCGGCCTTGCACAT	300/300
AV610889	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	GTTGCTGTAGCCGAATTCATTG	CACTCACTCTTCTACCTTCGATGCT	300/300
NM_174133	*UBB*	*Bos taurus* polyubiquitin (LOC281370), mRNA	AGATCCAGGATAAGGAAGGCAT	GCTCCACCTCCAGGGTGAT	300/300
NM_001046551	*CXCL10*	*Bos taurus* chemokine (C-X-C motif) ligand 10	TCGAGATTATTGCCACAATGA	CTTTGCTTGTTAATTGCTTTCAG	900/900
BF652431	*MSTN*	Myostatin	AGGAGAGATTTTGGGCTTGAT	TCCAATCCCATCCAAAAGCT	900/900
NM_174366.1	*ISG15*	Interferon-stimulated protein, 15 kDa	GACCTGACGGTGAAGATGCT	TGATCTTCTGGGCGATGAAC	900/900
NM_205793	*DGAT2*	*Bos taurus* diacylglycerol O-acyltransferase homolog 2 (mouse) (DGAT2), mRNA	AAGACTGCCCTTCCATCATG	CGGTAGTTGGGCAGCTCATA	300/300
NM_174313	*FABP*	Fatty acid binding protein (heart) like	AAATTCTCCTGGGGTCAGGT	GCCTTGGCTCTGCTTTATTG	300/300
NM_174292.2	*CRYGS*	Crystallin, gamma polypeptide 8	AAGACTGCCCTTCCATCATG	CGGTAGTTGGGCAGCTCATA	300/300

[a] Primer concentrations and sequence information for the primers are given in the 5′ to 3′ direction.

TABLE 2. Entrez gene symbol, Affymetrix probe ID, gene description, *P* value and fold change of DEG with greater than 1.5 fold difference in the endometrium of pregnant heifers with high and normal progesterone on Days 5, 7, 13, and 16.[a]

Entrez gene symbol	Affymetrix probe ID	Gene description	*P* value	Fold change
Day 5				
IBSP	Bt.568.1.S1_at	Integrin-binding sialoprotein (bone sialoprotein, bone sialoprotein II)	0.006	4.540
LOC782061	Bt.23094.3.S1_s_at	Similar to AKR1C1 protein	0.000	3.670
MSTN	Bt.8967.1.S1_at	Myostatin	0.035	3.280
MSTN	Bt.605.1.S1_at	Myostatin	0.035	3.040
DGAT2	Bt.23550.1.S1_at	Diacylglycerol O-acyltransferase homolog 2 (mouse)	0.021	2.870
LOC782061	Bt.23094.3.S1_at	Similar to AKR1C1 protein	0.000	2.230
	Bt.29191.1.A1_at		0.044	−1.530
SULT2B1	Bt.13420.1.S1_at	Sulfotransferase family, cytosolic, 2B, member 1	0.035	−1.770
	Bt.6905.1.S1_at		0.012	−1.850
LOC507436	Bt.4137.1.A1_at	Similar to Putative lymphocyte G0/G1 switch protein 2	0.021	−1.940
PRR5L	Bt.19824.2.S1_at	Proline rich 5 like	0.018	−2.190
LOC515128	Bt.16861.2.S1_at	Hypothetical LOC515128	0.000	−2.460
Day 7				
DGAT2	Bt.23550.1.S1_at	Diacylglycerol O-acyltransferase homolog 2 (mouse)	0.032	2.530
HPSE	Bt.8127.1.S1_at	Heparanase	0.001	2.210
RAB3B	Bt.5961.1.S1_at	RAB3B, member RAS oncogene family	0.032	1.900
	Bt.8127.1.S2_at		0.003	1.880
	Bt.5771.1.S1_at		0.002	1.870
	Bt.16687.1.A1_at		0.001	1.820
	Bt.7393.1.S1_at		0.032	1.820
	Bt.18861.1.A1_at		0.022	1.810
ASGR2	Bt.18330.2.S1_at	Asialoglycoprotein receptor 2	0.040	1.780
TMEM100	Bt.1787.2.S1_at	Transmembrane protein 100	0.048	1.780
MGC137099	Bt.14734.1.S1_at	Hepatitis A virus cellular receptor 1 N-terminal domain containing protein	0.007	1.750
	Bt.20580.1.S1_at		0.008	1.720
CEACAM1	Bt.10027.1.S2_at	Carcinoembryonic antigen-related cell adhesion molecule 1	0.007	−1.510
LOC526200	Bt.23276.1.S1_at	Similar to AIM1	0.025	−1.540
	Bt.21655.1.S1_at		0.033	−1.550
LOC526200	Bt.23276.2.S1_at	Similar to AIM1	0.007	−1.570
	Bt.6905.1.S1_at		0.026	−1.570
PRC1	Bt.20428.2.S1_a_at	Protein regulator of cytokinesis 1	0.047	−1.600
LOC539374	Bt.27193.1.A1_at	Similar to Family with sequence similarity 43, member A	0.038	−1.690
SERPINA5	Bt.2712.1.S1_at	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5	0.025	−1.720
LOC785602	Bt.3225.1.S1_at	Similar to glucose 6-phosphate translocase	0.006	−1.750
LOC539374	Bt.27193.2.S1_at	Similar to Family with sequence similarity 43, member A	0.007	−1.800
GCA	Bt.15419.1.S1_at	Grancalcin	0.025	−1.810
DRAM	Bt.26851.1.S1_at	Damage-regulated autophagy modulator	0.014	−1.820
CDCA4	Bt.8091.1.S1_at	Cell division cycle associated 4 LOC540850 to SYT7, synaptotagmin VII	0.000	−1.830
CORO2A	Bt.17910.2.S1_at	Coronin, actin binding protein, 2A	0.001	−1.910
GAT	Bt.25272.1.A1_at	Putative glycine-N-acyltransferase	0.031	−1.960
LOC526200	Bt.6504.1.S1_at	Similar to AIM1	0.006	−1.970
LOC540850	Bt.22274.1.S1_at	Similar to synaptotagmin VIIe	0.000	−2.150
UNC93A	Bt.28287.1.S1_at	unc-93 homolog A (C. elegans)	0.036	−2.160
ELL3	Bt.1565.1.S1_at	Elongation factor RNA polymerase II-like 3 MGC151920 to SLC6A20, solute carrier family 6 (proline IMINO transporter), member 20	0.007	−2.210
CORO2A	Bt.17910.1.A1_at	Coronin, actin binding protein, 2A	0.001	−2.320
CORO2A	Bt.28800.1.S1_at	Coronin, actin binding protein, 2A	0.001	−2.480
SVS8	Bt.232.1.S1_at	Seminal vesicle secretion 8	0.012	−2.610
FABP	Bt.4758.1.S1_at	Fatty acid binding protein (heart) like	0.007	−3.350
MGC151920	Bt.28747.1.S1_at	Similar to neurotransmitter transporter RB21A	0.007	−3.360
Day 13				
	Bt.18020.1.A1_at		0.014	−1.630
MRPS36	Bt.25235.1.A1_at	Mitochondrial ribosomal protein S36	0.003	−1.810
LOC614107	Bt.28139.1.S1_at	Similar to hexokinase II	0.038	−2.330
	Bt.2025.1.A1_at		0.003	−3.560
Day 16				
C15H11ORF34	Bt.6410.1.A1_at	Chromosome 11 open reading frame 34 ortholog	0.024	3.750
LOC514812	Bt.27333.1.S1_at	Cytokeratin 19	0.022	1.640
	Bt.17773.2.A1_at		0.022	1.570

[a] For full lists of all genes see Supplemental Table S1, A–D.

Temporal Changes in Endometrial Gene Expression Occur Throughout Early Pregnancy

To elucidate temporal changes in endometrial gene expression during early pregnancy, we compared gene expression patterns between Day 5 vs. Day 7, Day 7 vs. Day 13, and Day 13 vs. Day 16. In PH heifers, analysis of the GO terms associated with temporal changes in DEGs showed that genes involved in transport and cell cycle decreased, while genes involved in carbohydrate metabolic process and signal transduction increased as pregnancy progressed (Fig. 3, A–C). In PN heifers, there was a temporal decrease in DEGs

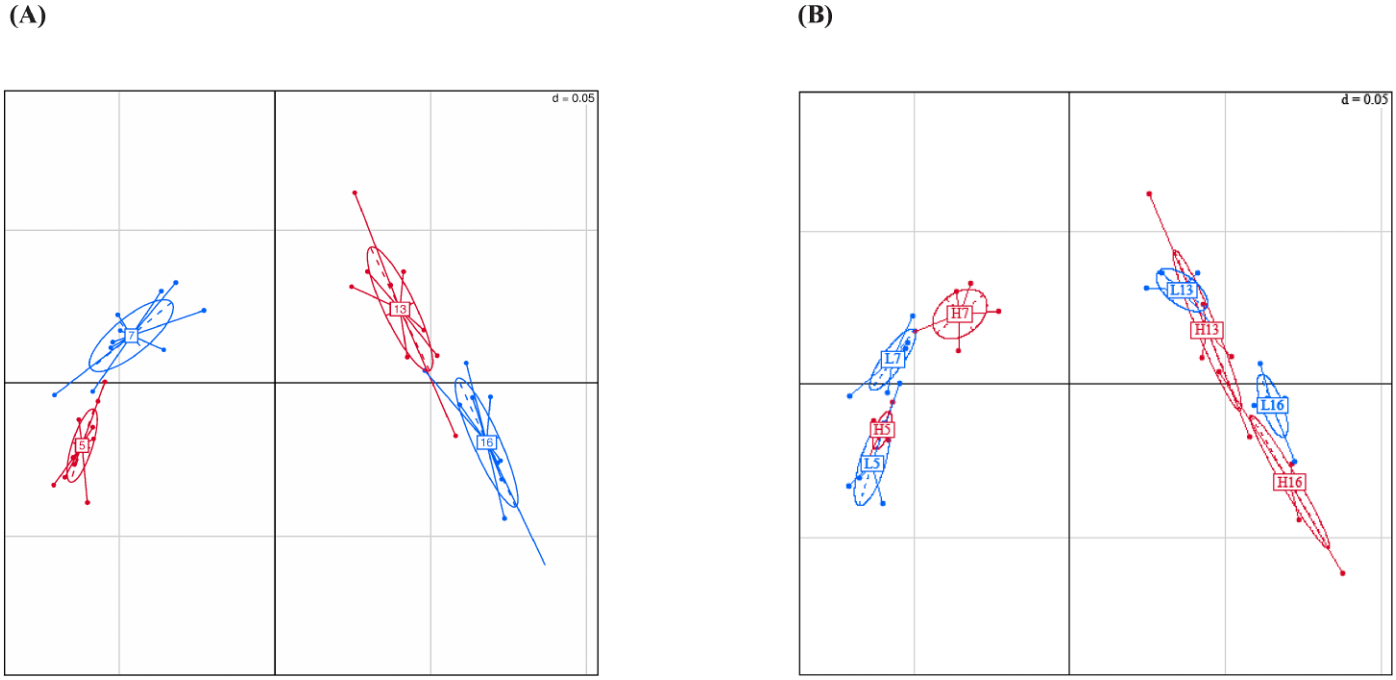

FIG. 2. Two-dimensional correspondence analysis. **A**) Plot distribution of segregation of transcription profiles for 40 endometrial samples into expression profile clusters corresponding to Days 5, 7, 13, and 16. **B**) Effect of high P4 on endometrial gene expression profile clusters for these same days. H represents high concentrations of P4 and L represents normal concentrations of P4.

associated with transcription and an increase in genes associated with transport, lipid metabolic process, and multicellular organismal development (Fig. 3).

In PN heifers, there were 1160 DEGs ($P < 0.05$) between Day 5 (8- to 16-cell stage) and Day 7 (morula/blastocyst stage) (Supplemental Table S3). GO terms that were overrepresented from this list of DEGs were associated with two main categories: 1) cellular localization, including intracellular transport, protein transport, and protein localization, and 2) metabolism, including glycerol, protein catabolic, and cellular and macromolecule metabolic processes (Supplemental Table S4).

The largest temporal alteration in endometrial gene expression occurred between Day 7 (zona-enclosed blastocyst) and Day 13 (hatched ovoid conceptus), with 2313 DEGs up-regulated and 2108 DEGs down-regulated ($P < 0.05$) (Supplemental Table S5). Seventy-six GO terms for biological processes were overrepresented (Supplemental Table S6), including protein transport and localization, as well as genes associated with the generation of precursor metabolites and energy, glucose metabolism and fatty acid biosynthetic process, and response to endogenous stimulus.

There were 426 DEGs between Day 13 and Day 16 of pregnancy ($P < 0.05$) (Supplemental Table S7). GO analysis indicated that 48 GO terms for biological processes were overrepresented, including response to stimulus, regulation of antiviral response to host, glucose transport, and translational initiation (Supplemental Table S8).

In PH heifers, the numbers of DEGs between Day 5 vs. Day 7 and Day 7 vs. Day 13 were comparable to those in PN heifers (Figure 4 and Supplemental Tables S9–S14). In contrast, there were twice as many DEGs between Day 13 and Day 16 in PH heifers compared with PN heifers, and 13 of 48 overrepresented GO terms were associated with immune response in PH heifers.

Temporally Expressed Gene Clusters Unique to PH Heifers Are Implicated in Advancement of Conceptus Elongation

In an attempt to identify which genes or groups of genes in the endometrium associated with high P4 may contribute to advanced conceptus elongation, the lists of DEGs across days in PN heifers were compared with those in PH heifers (Supplemental Tables S15 and S16). This comparison revealed that, irrespective of the P4 status of the heifers, a large number of genes were differentially expressed in PH heifers and PN heifers. Between Day 5 and Day 7, there were 855 and 850 DEGs in PH heifers and PN heifers, respectively. In PH heifers between Day 5 and Day 7, the primary overrepresented GO terms were involved in intracellular transport and cell cycle (Supplemental Tables S15 and S16). Between Day 7 and Day 13, there were 1067 and 2193 DEGs in PH heifers and PN heifers, respectively, with 2228 DEGs in both categories and no sole category of GO terms overrepresented in one treatment group compared with the other (Supplemental Tables S17 and S18). Between Day 13 and Day 16, despite the presence of 204 DEGs in both PH heifers and PN heifers, there were 666 DEGs in PH heifers compared with 222 DEGs in PN heifers, and the primary overrepresented GO terms from these 666 DEGs included those associated with positive regulation of inflammatory response, immune response, and embryo implantation (Supplemental Tables S19 and S20).

P4 Supplementation Advances Temporal Changes in Endometrial Gene Expression

To address the question of whether heifers with high P4 have advanced endometrial gene expression, we compared endometria from PH heifers on a specific day of pregnancy with endometria from PN heifers at the next stage of pregnancy (e.g., Day 5 PH heifers compared with Day 7 PN heifers [Fig. 4]). Between Day 5 PH heifers and Day 7 PN heifers, there

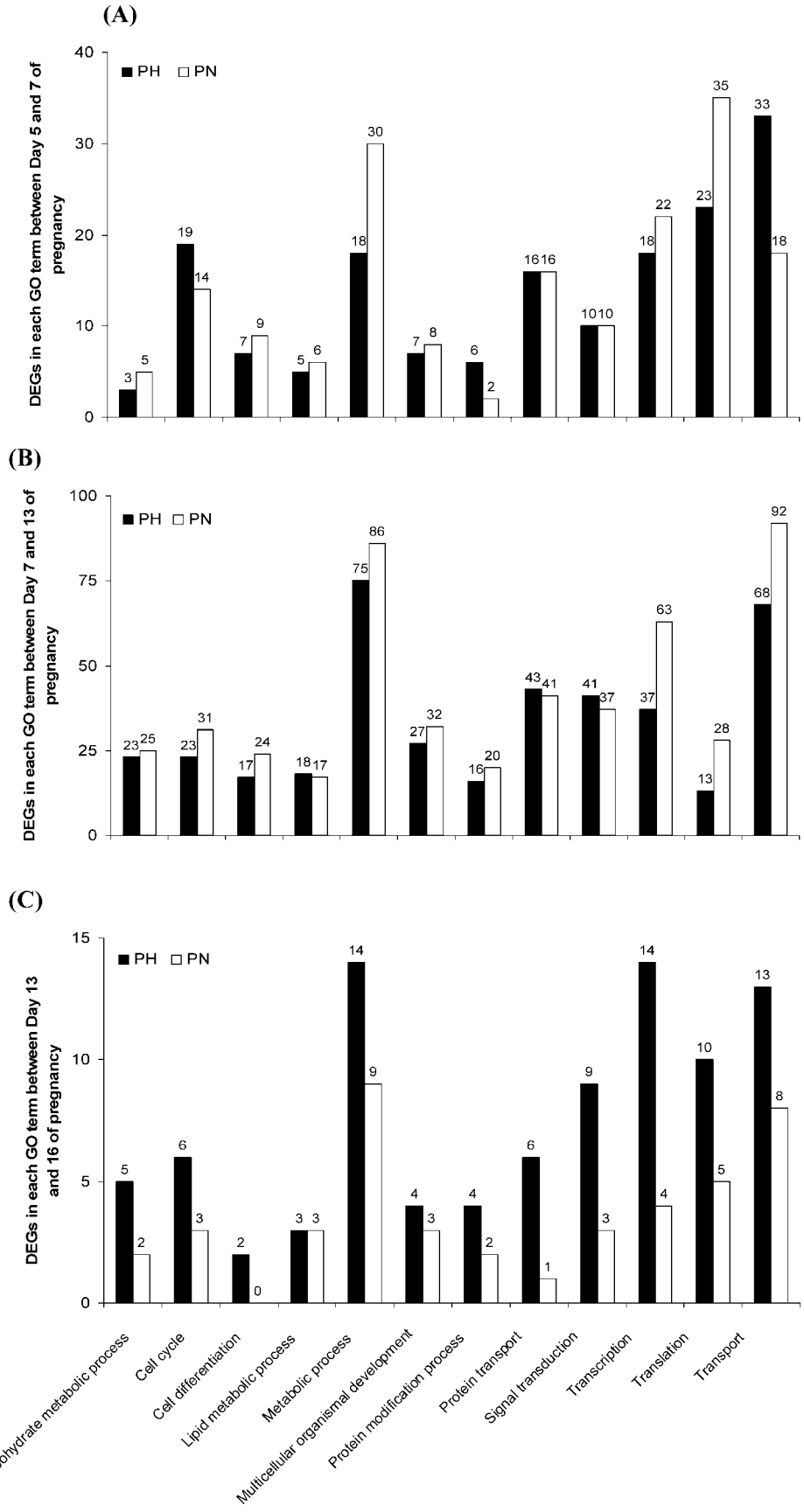

FIG. 3. Bar graph representing GO slim terms and the numbers of DEGs associated with each term between Day 5 and Day 7 (**A**), Day 7 and Day 13 (**B**), and Day 13 and Day 16 (**C**) in PH heifers and PN heifers. GO slim terms are only included when five or more DEGs are associated with the term in at least one category.

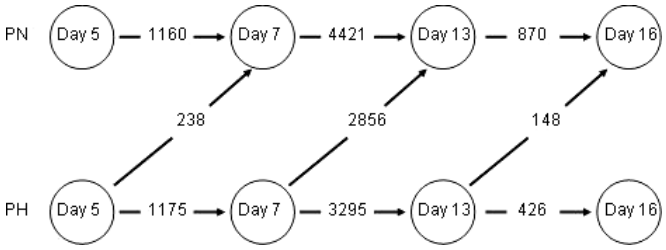

FIG. 4. Numbers of DEGs that are temporally regulated in the endometrium of PH heifers and PN heifers between Day 5 and Day 7, Day 7 and Day 13, and Day 13 and Day 16. Also shown are the numbers of DEGs indicating that P4 supplementation advanced endometrial gene expression between a Day 5 PH heifer vs. Day 7 PN, Day 7 PH vs. Day 13 PN, and Day 13 PH vs. Day 16 PN ($P < 0.05$). See supplemental data for lists of genes.

were 238 DEGs, with a large proportion involved in the GO terms of protein metabolic process, protein folding, and polymerization, as well as cellular macromolecule metabolic processes (Supplemental Tables S21 and S22). Comparison of Day 7 PH heifers with Day 13 PN heifers revealed the largest number of DEGs (n = 2856) (Supplemental Table S23), which were overrepresented in 89 GO categories, with the largest number of genes associated with metabolic processes (Supplemental Table S24). In contrast, Day 13 PH heifers compared with Day 16 PN heifers only revealed 148 DEGs, with few genes associated with overrepresented GO terms (Supplemental Tables S25 and S26).

Quantitative Real-Time PCR Validation of Microarray Data

Using quantitative real-time PCR, six genes (*MSTN*, *DGAT2*, *FABP*, *CRYGS*, *CXCL10*, and *ISG15)* were validated at all stages in all groups, including within-day comparisons (i.e., 10 comparisons for each gene) (Figs. 5 and 6). The quantitative real-time PCR expression results (mean ± SEM) mirrored the microarray data with a very high degree of fidelity (>95% agreement). Within a specific day of pregnancy, expression of *DGAT2* was higher in PH heifers compared with PN heifers on Day 7 ($P < 0.05$). Across time, *DGAT2* expression in PH heifers increased from Day 5 to Day 7 ($P < 0.05$), remained high on Day 13, and then decreased on Day 16 ($P < 0.05$). In PN heifers, *DGAT2* expression increased sequentially between Day 5 and Day 7 and between Day 7 and Day 13 and decreased on Day 16 ($P < 0.05$) (Fig. 5A). There were no significant differences in *DGAT2* when the advancement comparisons were made ($P > 0.05$).

CRYGS expression was only different between PH heifers and PN heifers on Day 13 ($P < 0.05$) (Fig. 5B). *CRYGS* expression across time increased between Day 5 and Day 7 in both PH heifers and PN heifers. In PH heifers, there was a decrease in expression on Day 16 compared with Day 13 ($P < 0.05$). In PN heifers, the decrease occurred earlier on Day 13 compared with Day 7 ($P < 0.05$). On Day 7, *FABP* expression was higher in PN heifers compared with PH heifers, but on Day 13 there was a decrease in PN heifers compared with PH heifers ($P < 0.05$) (Fig. 5C). Across time in PH heifers, there was a decrease on Day 7 compared with Day 5 and a subsequent increase by Day 13 ($P < 0.05$). In PN heifers, *CRYGS* expression decreased on Day 13 compared with Day 7 and increased on Day 16 compared with Day 13 ($P < 0.05$). *FABP* expression was only different between Day 13 PH heifers vs. Day 16 PN heifers ($P < 0.05$).

For *MSTN* expression, there was an increase in expression in PH heifers compared with PN heifers on Day 5 only ($P < 0.05$) (Fig. 6A). There was a significant decrease in expression on Day 7 compared with Day 5 in PH heifers, with an increase on Day 13 compared with Day 7. *MSTN* expression in PN heifers increased on Day 13 compared with Day 7 only ($P < 0.05$). There was also a significant difference in expression between Day 5 PH heifers vs. Day 7 PN heifers and between Day 7 PH heifers vs. Day 13 PN heifers. Both *ISG15* and *CXCL10* expression was significantly increased on Day 16 vs. Day 13 in both PH heifers and PN heifers, with only *CXCL10* expression significantly increased in PH heifers compared with PN heifers on Day 16 ($P < 0.05$) (Fig. 6, B and C).

DISCUSSION

The main findings from the present study are the following: 1) P4 supplementation alters endometrial gene expression at critical stages of periimplantation embryo development; 2) significant temporal changes in the transcriptional profile of the endometrium coincide with different developmental stages of the conceptus in early pregnancy; 3) these changes are affected by elevated P4, and both contribute to and are a consequence of advanced development of the conceptus; and (4) P4 supplementation during early pregnancy advances endometrial gene expression in cattle [10].

The factor that contributed most to altered endometrial gene expression was day of pregnancy. As shown in Figure 2A, Day 5 and Day 7 samples (prehatching-stage embryos) clustered together, and Day 13 and Day 16 samples (posthatching initiation of and elongating conceptuses) clustered together, regardless of the P4 status of the heifer. The importance of day of pregnancy was further highlighted when endometria collected within short intervals (e.g., Day 5 vs. Day 7) were compared, which resulted in small numbers of DEGs, in contrast to comparisons between more divergent time points (e.g., Day 7 vs. Day 13), which resulted in the largest number of DEGs (Fig. 4).

Published evidence obtained using Affymetrix chips indicates that microarray data are robust and reliable [22, 23]. As expected, comparison of results from the microarray and quantitative real-time PCR analyses revealed a very high degree (>95%) of fidelity. Insertion of a PRID on Day 3 resulted in a significant increase in P4 concentrations in serum by Day 3.5, which was maintained until Day 7 [10]. This window of increased P4 corresponds to the period when elevated levels of P4 have been associated with increased embryonic development and survival [1, 6–8]. In the present study, P4 supplementation induced changes at all four stages of pregnancy studied, but the largest number of DEGs coincided with the time when P4 profiles were divergent for the longest period (i.e., up to Day 7 of pregnancy) (Table 2) [10].

Elevated P4 concentrations resulted in large fold change differences in only a few genes, which is consistent with previous findings [22]. Regulation of a number of these genes has been associated with P4-dominant environments in cattle (*DGAT2*) and in humans (*MSTN*) [18, 24, 25]. Given that *DGAT2* catalyzes the final step in the formation of triglyceride to acylcoenzyme A [26] and that triglyceride is a potential energy source up to the blastocyst stage in cattle [27], a P4-induced earlier increase in *DGAT2* expression may increase availability of triglyceride as an energy source for the developing conceptus. *MSTN* is detectable in the P4-dominant secretory phase in human endometrial culture in vitro [24]. *Mstn*-null mice display reduced fat accumulation and abnormal glucose metabolism [28], and in vitro treatment of human term placenta resulted in increased deoxyglucose uptake [29]. Thus, P4 induction of *MSTN* may increase glucose secretion into

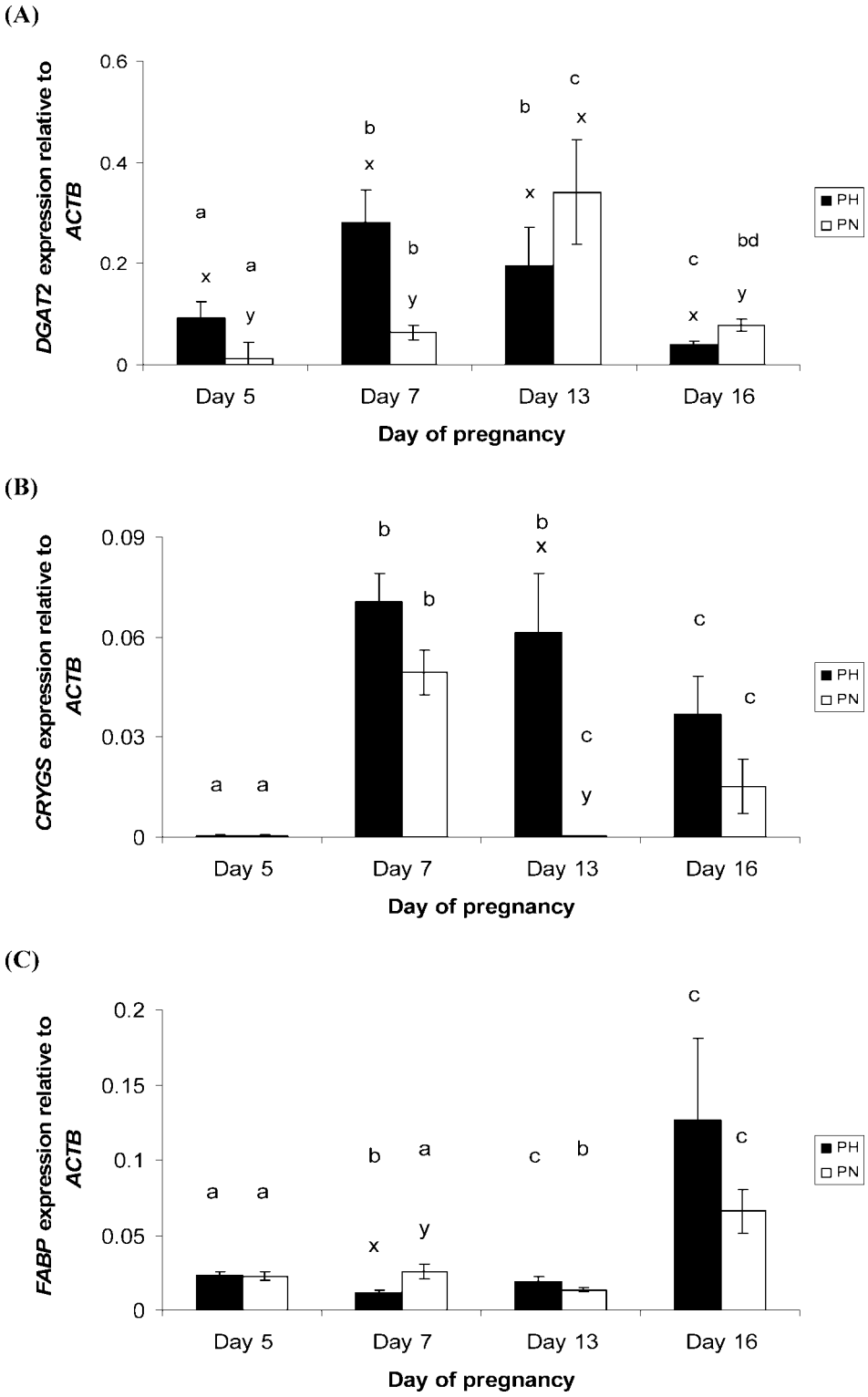

FIG. 5. Quantitative real-time PCR analysis of *DGAT2* (**A**), *CRYGS* (**B**), and *FABP* (**C**) gene expression relative to *ACTB* (the normalizer) for PH heifers and PN heifers. Expression values are presented as the mean ± SEM (n = 5 per treatment per day). The x,y indicates a significant difference between groups within a time point, and a,b,c,d indicates significant differences within a treatment group across time ($P < 0.05$).

histotroph, contributing to the advanced development of the conceptus after hatching. The regulation of genes associated with energy production or sources that can be secreted into histotroph indicates one method whereby P4 may advance conceptus development after hatching.

Analysis of the GO terms associated with the DEGs that change temporally indicated a number of distinct differences between PH heifers and PN heifers (Fig. 3). In PH heifers, there is a decrease in DEGs involved in transport between Day 13 and Day 16 compared with PN heifers, where the number of genes increases with an inverse change in carbohydrate and lipid metabolic processes. This suggests that in the PH heifers the protein products that are required in the lumen to advance conceptus development have been transported into the lumen at an earlier stage than what occurs in a normal P4 environment. Further weight is given to this argument by the comparison to determine if P4 supplementation advances endometrial gene expression (Fig. 4). The total number of DEGs that occurs when endometria from different stages of conceptus development are compared is much higher than when endometria from

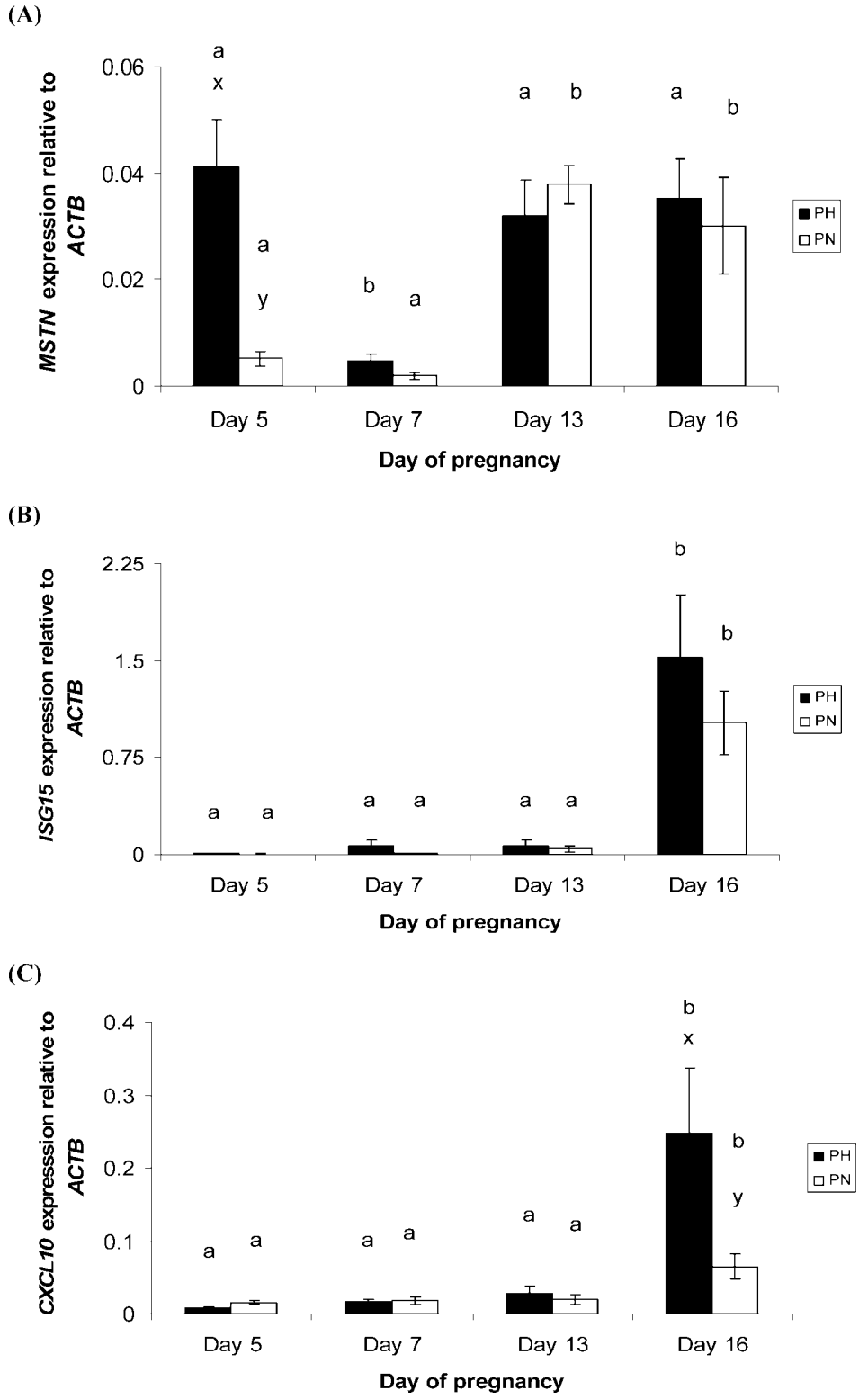

FIG. 6. Quantitative real-time PCR analysis of *MSTN* (**A**), *ISG15* (**B**), and *CXCL10* (**C**) gene expression relative to *ACTB* (the normalizer) for PH heifers and PN heifers. Expression values are presented as the mean ± SEM (n = 5 per treatment per day). The x,y indicates a significant difference between groups within a time point, and a,b,c,d indicates significant differences within a treatment group across time ($P < 0.05$).

PH heifers are compared with endometria from PN heifers from the stage ahead (Fig. 4). When expression of genes that are P4 regulated and may contribute to histotroph composition (*MSTN*, *FABP*, and *DGAT2*) are compared, there is no significant difference in their expression levels.

Analysis of the overrepresented GO terms between Day 5 and Day 7 revealed temporal regulation of genes involved in triglyceride synthesis such as *DGAT2*, *MGOAT1*, and *MVK* (which are required for cholesterol biosynthesis [30]) and *ACSL5* [31] and *SLC27A6* (which are involved in acylcoenzyme A synthesis [32]). None of these genes have a significantly different fold change in expression between Day 5 PH heifers compared with Day 7 PN heifers, indicating that the advancement of potentially advantageous genes has occurred earlier in PH heifers, which may contribute to the larger conceptus recovered on Day 13 and Day 16.

The results presented herein indicate distinct temporal changes in gene expression between Day 7 and Day 13. These

can be broadly associated with distinct biological processes involved in mitochondrial electron transport and ATP production, cytoskeletal remodeling/cell cycle progression, and transport and synthesis of nucleotides, proteins, and energy sources. Members of this transport ATP synthase family that are responsible for the transport of sodium, potassium, and calcium into and out of cells at the expense of ATP production [33] were down-regulated on Day 13, while some members of the ATP synthase family that are associated with ATP production [34] were up-regulated. This suggests that a reduction in sodium/potassium transport is associated with increased ATP production to meet the energy requirements of the conceptus and/or uterus during early pregnancy. When the question of advancement in endometrial gene expression is addressed, we have shown that there are still a large number of DEGs between Day 7 PH heifers and Day 13 PN heifers. There are fewer differences between Day 7 PH heifers and Day 13 PN heifers than between Day 7 PH heifers and Day 13 PH heifers (Fig. 4). Moreover, the fact that these genes involved in ATP production are still differentially expressed in the Day 7 PH heifers vs. Day 13 PN heifers comparison indicates that this is a required process throughout the early stages of pregnancy, which is not surprising given that the correspondence analysis revealed that the source of greatest variation in the transcriptional profile of the endometrium is day of pregnancy (Fig. 2A).

Our findings also reveal progressive up-regulation of different interferon-stimulated genes (ISGs) during pregnancy by Day 13 compared with Day 7 (e.g., *IFI6*, *IRF9*, *IFITM1*, *MX1*, and *OAS1*) and by Day 16 compared with Day 13 (e.g., *ISG15*, *IRF9*, *MX2*, and *STAT1*). The sequential increase in expression of these genes indicates increased responsiveness of the endometrium to IFNT on Day 13 compared with Day 7, and this responsiveness increases further between Day 13 and Day 16 of pregnancy. The comparison between Day 13 PH heifers and Day 16 PN heifers revealed few DEGs, a number of which are ISGs. Although we have shown that the conceptus is larger in PH heifers compared with PN heifers, it is not surprising that expression of some ISGs is elevated in Day 16 PN heifers compared with Day 13 PH heifers given the rapid elongation of the conceptus trophectoderm from an ovoid conceptus on Day 13 to an elongated conceptus on Day 16 [10].

Another approach to elucidate which genes or groups of genes might be responsible for advancing conceptus elongation, DEGs across days in PN heifers were compared with those in PH heifers. This generated a temporal gene expression signature in PH heifers that may contribute to the advanced conceptus development apparent on Day 13 and Day 16. Between Day 5 and Day 7, a large number of DEGs are involved in protein transport and localization. Although a number of these genes are involved in intracellular transport of proteins that may not contribute directly to the composition of histotroph, they are involved in trafficking of potential components of histotroph [35] (e.g., solute carrier family members are required for secretion of histotroph). Moreover, increases in glucose in ovine histotroph during early pregnancy are associated with increases in mRNAs for both facilitative and sodium-dependent glucose transporters in the uterine endometrium, some of which are induced by P4 and stimulated further by IFNT [36, 37]. Because histotroph is essential for conceptus development and (in turn) establishment of pregnancy [14, 38], P4 supplementation may increase gene expression and subsequent trafficking of gene products that stimulate conceptus development. Between Day 7 and Day 13, a major shift in uterine gene expression occurs with the loss of P4 receptors in uterine luminal epithelia [39]. This loss of P4 receptor is associated with the induction of numerous genes associated with cell adhesion [13].

Gene regulation in response to elevated P4 between Day 13 and Day 16 may be a response of the endometrium to earlier down-regulation of P4 receptor in uterine epithelia and advanced conceptus development, as P4 profiles are not different at these stages of conceptus development [10]. Further support for this theory is provided by the fact that a number of ISGs were up-regulated on Day 16 compared with Day 13 of pregnancy, regardless of P4 status. These include *ISG15*, *MX2*, and *LOC781857* (similar to interferon-induced protein 44, for which expression patterns are associated with pregnancy status [40–42]).

In conclusion, this study describes temporal changes in the transcriptional profile of the endometrium from Day 5 to Day 16 of early pregnancy for the first time (to our knowledge) and reveals how endometrial genes are regulated by circulating levels of P4. The analysis demonstrates that P4 supplementation advances endometrial gene expression, particularly genes associated with energy sources or contributors to histotroph, which may contribute to advanced conceptus development on Day 13 and Day 16 [36, 37]. This work provides P4-regulated endometrial gene expression signatures that are associated with advanced conceptus development and represents the basis for future studies examining the transcriptional and proteomic regulation of early pregnancy.

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