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Transcriptional Profiling of Equine Conceptuses Reveals New Aspects of Embryo-Maternal Communication in the Horse[1]

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ABSTRACT

Establishment and maintenance of pregnancy are critically dependent on embryo-maternal communication during the preimplantation period. The horse is one of the few domestic species in which the conceptus-derived pregnancy recognition signal has not been identified. To gain new insights into the factors released by the equine conceptus, transcriptional profiling analyses of conceptuses retrieved 8, 10, 12, and 14 days after ovulation were performed using a whole-genome microarray. Selected array data were confirmed using quantitative PCR, and the expression of proteins of interest was confirmed using immunohistochemistry and Western blotting. Gene ontology classification of differentially regulated transcripts underlines the ongoing embryo-maternal dialogue. Transcript showing higher expression levels as conceptus' development proceeds mainly localizes to the extracellular environment, thereby having the potential to act upon the uterine environment. Genes involved in the positive regulation of the immune system are enriched among transcripts displaying decreased expression, reflecting the need of the semiallograft conceptus to be protected from the immune system. A subset of differentially expressed genes, such as *BRCA1* and *FGF2*, has previously been described to be expressed by early stages of embryonic development, whereas other transcripts are apparently unique to equine conceptuses, as their expression has not been reported in other species. These transcripts include fibrinogen subunits, the expressions of which were confirmed at the mRNA and protein level. Furthermore, results indicate the counteraction of trophoblast invasion, and that the conceptus appears to regulate changes in sialic acid content of its capsule, an event suggested to be essential for successful establishment of pregnancy.

conceptus, early development, embryo, embryo-maternal interaction, fibrinogen, gene regulation, microarray, pregnancy, trophoblast

INTRODUCTION

Early conceptus development, implantation and maintenance of pregnancy are critically dependent upon a precisely orchestrated interaction between the conceptus and the uterine environment. This maternal-conceptus interaction, if successful, prompts continued progestin support, leading to a receptive uterine environment, which, in turn, is essential for the conceptus' development. The early conceptus signals its presence through biochemical processes that interrupt estrous cyclicity and maintain pregnancy. ''Maternal recognition of pregnancy'' (MRP), a phrase coined by Roger Short in 1963 [1], is commonly used to describe the continuum of events leading to maintenance of gestation after successful fertilization and descent of the developing conceptus into the uterus. In a more narrow sense, the expression MRP is used to refer to the physiological processes by which the lifespan of the corpus luteum is prolonged. These processes are best understood in domestic ruminants and other ungulates, such as the pig. Interferon tau has been identified as a conceptus-derived paracrine factor exhibiting antiluteolytic properties in ruminants, while conceptus-derived estrogens represent the primary pregnancy recognition signal in pigs [2, 3]. The horse is one of the few domestic species in which the conceptus-derived pregnancy recognition signal has not been identified and equids appear to be distinct from ruminants and pigs in the signal or signals being used for MRP. Horses exhibit some unusual features during early pregnancy that likely contribute to pregnancy recognition. For example, the spherical equine conceptus migrates continuously throughout the uterine lumen between Days 9 and 16 after ovulation [4]. Restriction of conceptus movement results in luteolysis, with subsequent failure of pregnancy maintenance [5]. Equine conceptuses also produce substantial amounts of estrogens and prostaglandins [6–8]; however, their precise roles during early pregnancy remain unclear. Experiments attempting to prove that embryo-derived estrogens are responsible for extension of corpus luteum function have been inconclusive [9, 10].

Microarray-based transcriptional profiling of both endometrial and embryonic tissues has been applied in various species to gain further insight into the molecular events underlying embryo-maternal interactions during MRP [11–13]. The objective of the present investigation was to profile mRNA transcripts of Day-8, -10, -12, and -14 equine conceptuses using a custom-designed whole-genome microarray. Time points were chosen to analyze conceptuses retrieved before the onset of MRP (i.e., Day 8), during the onset of MRP (i.e., Day 10), and during MRP (i.e., Days 12 and 14). Array data for selected genes were validated using quantitative RT-PCR (qRT-PCR) and expression of proteins of interest was confirmed using immunohistochemistry (IHC) and Western blotting.

MATERIALS AND METHODS

Animals and Tissue Collection

All animal procedures were completed in accordance with and with the approval of the Institutional Animal Care and Use Committee at the University of Kentucky. The luteal phase of the estrous cycle of mares was shortened by the administration of 10 mg of a prostaglandin analogue, dinoprost (Lutalyse; Pfizer, New York, NY). After induction of luteolysis, the animals were examined daily for follicular development, development of

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uterine edema, as well as cervical and uterine tone. In the absence of a distinct corpus luteum, the presence of a follicle at least 35 mm in diameter, pronounced uterine edema, and diminished uterine and cervical tone, each mare received 2,500 IU of human chorionic gonadotropin (hCG) (Chorulon; Intervet, Millsboro, DE) as an ovulation-inducing agent, and was simultaneously inseminated. For insemination, a minimum of 500×10^6 progressively motile sperm were used; semen was collected the day of insemination. The collected semen was extended with Equipro with ticarcillin (Minitube, Mount Horeb, WI) to a final concentration of 50×10^6/ml. Mares were examined daily by ultrasonography for signs of ovulation. If ovulation had not occurred within 48 h after insemination, another artificial insemination was performed. Conceptuses were recovered 8, 10, 12, and 14 days after ovulation through transcervical flush, snap frozen in liquid nitrogen, and stored at −80°C until further processing.

Isolation of RNA

Cellular RNA longer than 200 nucleotides was isolated from four conceptuses per developmental stage using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was analyzed for quality by determining the RNA integrity number (RIN) using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and was quantified via spectrophotometry using a NanoDrop ND-1000 (Agilent Technologies). Samples with a RIN of 8.0 or greater, a 260:280 ratio of 1.95 or greater, and a 260:230 ratio of 2.0 or greater were used for analysis.

Array Hybridization

Fluorescent cRNA was generated using the Low RNA Input Linear Amplification Kit (Agilent Technologies). In brief, total RNA (200 ng) was reverse transcribed to cDNA using primers containing a T7 RNA polymerase site, so that a T7 sequence was introduced in the resulting cDNA. T7 RNA polymerase-driven in vitro transcription was then used for the preparation and labeling of RNA with Cy3 or Cy5 dye. The fluorescent cRNA was purified using Qiagen RNeasy Mini kit, quantified using a NanoDrop ND-1000 (Agilent Technologies), and an equal amount (825 ng) of Cy3- and Cy5-labeled cRNA probes were hybridized on the custom array. Only samples that showed a specific activity equal to or greater than 8.0 pmol Cy3 or Cy5 per microgram of cRNA were used for array hybridization. Individual Day-8 conceptuses ($n = 4$, labeled with Cy3) were paired with individual Day-10 ($n = 4$, labeled with Cy5), Day-12 ($n = 4$, labeled with Cy5), and Day-14 ($n = 4$, labeled with Cy5) conceptuses, resulting in a total of 12 hybridizations. An equine-specific gene expression microarray containing 43 803 features previously constructed by the authors was used [13]. Hybridization was carried out overnight at 65°C for 17 h, and hybridized slides were washed using the Gene Expression Wash Buffer kit, followed by Stabilization and Drying solution (Agilent Technologies). DNA Microarray Scanner was used for image acquisition (Agilent Technologies).

Analysis of Array Data

Feature Extraction 10.7 Image Analysis Software (Agilent Technologies) was used for computation of feature intensities. Mean feature intensity corrected for the local background was used for further analysis, and is hereafter referred to as raw data. Raw data were imported into JMP Genomics 3.0 (Cary, NC) and log base 2 transformed. Quality of raw data was assessed using Distribution Analysis and Correlation and Principal Component Analysis functions in JMP Genomics. MA plots (a plot of the distribution of the red:green intensity ratio (''M'') plotted by the average intensity (''A'') were generated to visualize intensity-dependent ratio of raw data. Raw data were normalized using Locally Weighted Linear Regression (LOWESS). Mixed model analysis was performed to identify significantly differentially expressed genes, whereby stage of conceptus development was included as a fixed effect and array as random effect. An *F*-test on least-square means was used to estimate the significance of difference for each gene in each comparison where $P < 0.01$ was considered to be statistically different. Only genes showing a difference in signal intensity of at least 2-fold between Day-8 and Day-10, -12, or -14 conceptuses in all four biological replicates were considered to be differentially expressed. Of the transcripts identified as differentially expressed between the different stages of conceptus development, only those classified as ''well above background'' by the Feature Extraction software were kept for further analysis.

Based on the data for human orthologous genes, the Database for Annotation, Visualization, and Integrated Discovery was used to categorize the genes regarding their molecular function, cellular compartment, or the biological processes in which they are involved, respectively. Only significantly enriched categories were considered. Resulting data were supplemented with additional information from Entrez Gene (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene) and from the literature (http://www.ncbi.nlm.nih.gov/PubMed/).

Real-Time RT-PCR

Total RNA (200 ng) was treated with RNase-free DNase I (Ambion, Woodward Austin, TX) for 15 min at 37°C, heat denatured (75°C for 10 min), then reverse transcribed using High Capacity cDNA Reverse Transcription Kit and random hexamers (Applied Biosystems, Foster City, CA). Equine-specific primers for the selected transcripts were designed using Primer-BLAST (NCBI). Amplification efficiencies were similar between all primer sets used as determined using 1:10 serial dilutions of standard endometrial RNA samples. PCR products were sequenced to confirm identity of the amplicon. Real-time PCR was completed using 0.5 μl of cDNA andSYBR Green PCR Master Mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems) with the following cycling conditions: 95°C for 10 min; 45 cycles of 95°C for 15 sec and 60°C for 1 min; 55 to 95°C for dissociation. Each PCR was performed in triplicate. All reactions were automatically pipetted using the epMotion Automated Pipetting Systems (Eppendorf, Westbury, NY). Specificity of amplification was monitored by including non-reverse-transcribed RNA reactions for each sample, and by completing a dissociation analysis at the end of each real-time run to verify the amplification of a single product. Changes in gene expression were calculated by mean threshold cycle (C_T) and then normalized for the housekeeping gene 18S to generate delta (Δ) C_T values. A total of six presumptive reference genes had previously been tested with regard to stable expression levels, and *18S* was shown to be the most stably expressed transcript across the different stages of conceptus development (data not shown). Linreg (http://www.gene-quantification.de) was used to determine the efficiencies of each PCR reaction followed by efficiency correction of Ct values. Changes in abundance of specific transcripts relative to levels in Day-8 conceptuses were examined by calculating the fold-effect using the $\Delta\Delta C_T$ method [14]. ΔC_T values were compared using *t*-test analysis and a *P* value of 0.05 or less was considered statistically significant. Statistical analysis was carried out using SAS (SAS Institute, Cary, NC).

Immunohistochemistry

Conceptuses obtained through transcervical flush 14 days after ovulation were fixed in 4% formaldehyde for 24 h and then embedded in paraffin. Staining was performed on an automated staining system (Bond-MAX; Leica Biosystems, Newcastle Upon Tyne, UK) following the manufacturer's protocols. This involved an initial automated dewaxing and rehydration step, followed by heat-induced (100°C for 20 min) antigen retrieval using pH 8.8 ethylenediaminetetraacetic acid (EDTA)-based ready-to-use solution (Leica Biosystems). The slides were subsequently incubated with 3% hydrogen peroxide (5 min), optimally diluted primary antibody (30 min), a postprimary blocking reagent (to prevent nonspecific polymer binding) (8 min), horseradish peroxidase-labeled polymer (8 min), and diaminobenzidine substrate (10 min). All reagents were components of the Bond Polymer Refine detection system (Leica Biosystems).

Primary antibodies were diluted to optimal concentration (1:200) using Bond Primary Antibody Diluent (Leica Biosystems). Washing steps between each reagent were performed using Bond Wash Solution 10× Concentrate (Leica Biosystems) diluted to a 1× working solution with distilled water. Counterstaining was performed using hematoxylin. Sections were dehydrated and mounted with a cover slip. As negative controls, the primary antibody was substituted with serum block solution or normal serum from the species the primary antibody was raised in. The following polyclonal primary antibodies were used, relying on their cross-reactivity with the equine protein: rabbit anti-human fibrinogen alpha (FGA, sc-33580; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human fibrinogen beta (FGB, sc-33581; Santa Cruz Biotechnology), rabbit anti-human fibrinogen gamma (FGG, sc-33582; Santa Cruz Biotechnology), rabbit anti-human plasminogen activator inhibitor (PAI)-1 (sc-8979; Santa Cruz Biotechnology), and rabbit anti-human neuraminidase 2 (NEU2, Anti-NEU2 56-70; Sigma-Aldrich, St. Louis, MO).

Generation of Conceptus-Conditioned Medium

Conceptuses obtained through transcervical flush 14 days after ovulation were incubated in Dulbecco modified Eagle medium/F12 medium (Invitrogen) supplemented with Antibiotic-Antimycotic (Invitrogen) for 24 hours. Conceptus-conditioned medium (CCM) was then concentrated using Centrifugal Filter Units (Millipore, Billerica, MA). Concentrated CCM was stored at −80°C till further analysis.

TABLE 1. Number of genes differentially regulated between Day-8 and Day-10, Day-12, and Day-14 conceptuses, respectively.

Parameter	Differentially regulated	Up-regulated	Down-regulated
Day 8 vs. Day 10	355	262 (63%)	93 (65%)
Day 8 vs. Day 12	1211	806 (88%)	405 (90%)
Day 8 vs. Day 14	1144	635 (77%)	509 (59%)

Western Blotting

Detection by immunoblotting was carried out for FGA and FGG using protein isolated from conceptuses, yolk sac fluid, capsular material, and CCM using the same antibodies used for IHC. Total protein from conceptuses was recovered from flow-through of the RNeasy spin column through acetone precipitation. Four volumes of ice-cold acetone were added to the flow-through and stored at −20°C. Protein was precipitated through centrifugation at 12 000 × *g* for 10 min. The resulting pellet was resuspended in 1× Laemmli sample buffer (Bio-Rad, Hercules, CA). CCM, yolk sac, and capsular material were mixed with an equal volume of 2× Laemmli sample buffer. Samples were then incubated at 95°C for 4 min followed by electrophoresis on 15% SDS polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose (Bio-Rad) using an electroblotting apparatus (Bio-Rad). Membranes were incubated for 1 h in TBS (10 mM Tris-CHl, 150 mM NaCl) with 5% nonfat dry milk to block nonspecific binding, followed by incubation with primary antibody (diluted 1:400) overnight at 4°C. Following several wash steps with PBS with 0.1% SDS, binding of primary antibody was detected using horseradish peroxidase-conjugated mouse anti-rabbit immunoglobulin (diluted 1:10 000; Sigma-Aldrich) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). As negative control, the primary antibody was omitted and replaced with PBST containing 1% BSA.

RESULTS

Detection of Differentially Expressed Genes in Conceptuses Obtained 8, 10, 12, and 14 Days after Ovulation

Custom oligonucleotide microarrays were used to analyze differences in steady-state mRNA between Day-8 and Day-10, -12, and -14 conceptuses. RNA was isolated, fluorescently labeled, and analyzed after array hybridization. A total of 355, 1211, and 1144 transcripts were identified as differentially expressed between Day-8 and -10, Day-8 and -12, and Day-8 and -14 conceptuses, respectively. Transcripts with higher expression levels than Day-8 conceptuses are referred to as up-regulated, and those with lower expression levels than Day-8 conceptuses as down-regulated. Table 1 gives an overview of the number of transcripts differentially regulated and the percentage of genes thereof with known or inferred function. A total of 151 differentially expressed transcripts were common between Day-10, -12, and -14 conceptuses, and are listed in Table 2. Remaining transcripts (n = 1693) showing a statistically significant difference in only Day-10 (n = 110), -12 (n = 524), or -14 (n = 495) conceptuses, or showing a statistically significant difference in Day-10 and -12 (n = 66), Day-10 and -14 (n = 28), and Day-12 and -14 conceptuses (n = 470), respectively, are listed in the Supplemental Data (available at www.biolreprod.org). Figure 1 depicts the overlap of differentially expressed transcripts between Day-10, -12, and -14 conceptuses using a Venn diagram. The results for the gene ontology (GO) classification cellular component, molecular function, and biological process for up- and down-regulated transcripts are depicted in Figure 2. The majority of genes showing higher expression in Day-10, -12, and -14 conceptuses are consistent with cell surface events, such as transcripts falling into the cellular compartments ''extracellular region'' and ''intrinsic to plasma membrane.'' The biological processes in which up-regulated transcripts are involved reflect the response of the conceptus to various stimuli as well as lipid biosynthetic and fatty acid metabolic processes taking place. Down-regulated transcripts mainly localize to the nucleus, are involved in regulation of gene expression, cell division, and regulation of immune response.

Real-Time RT-PCR

To verify selected expression data obtained from the microarray analysis, RT-qPCR was performed on a subset of genes that showed a significant up- or down-regulation between Day-10, -12, and -14 conceptuses when compared with Day-8 conceptuses. Transcripts were selected based on their potential developmental significance and based on their fold change to include genes with a low and high fold change. Eleven up-regulated (aquaporin 5 [*AQP5*]; *NEU2*; *FGG*; *FGB*; *FGA*; CYP19A1 cytochrome P450, family 19, subfamily A, polypeptide 1 [*CYP19A1*]; fibroblast growth factor [*FGF*] 13; interferon alpha 1 [*IFNA1*]; insulin-like growth factor binding protein 6 [*IGFBP6*]; apolipoprotein B [*APOB*]; coagulation factor X, *F10*), and five down-regulated genes (secreted phosphoprotein 1 [*SPP1*]; P19 lipocalin [*P19*]; signal peptide, CUB domain, EGF-like 2 [*SCUBE2*]; solute carrier family 26, member 4 [*SLC26A4*]; serine peptidase inhibitor, Kazal type 7 [*SPINK7*]) were included in the analysis (Fig. 3). Difference in expression data obtained by microarray analysis were confirmed using this approach; all transcripts showed a statistically significant difference in expression when compared with levels in Day-8 conceptuses. The ratios obtained by RT-qPCR paralleled those obtained by array hybridization in 15 out of 16 transcripts; *SLC26A4*, which showed a significant down-regulation in the microarray analysis, showed only a mild trend toward down-regulation upon RT-qPCR. For highly expressed transcripts, such as *FGA*, *FGB*, and *FGG*, the fold change obtained by RT-qPCR analysis was higher than that obtained by microarray analysis. This discrepancy in fold change highlights the tendency of microarrays to show suppressed changes in expression due to their narrower dynamic range relative to RT-qPCR, a phenomenon most pronounced for highly expressed genes [15].

Immunohistochemical Localization of Selected Gene Products

IHC was performed using five selected up-regulated genes, *FGA*, *FGB*, *FGG*, *NEU2*, and *PAI1* (Fig. 4). *FGA*, *FGB*, and *FGG* were selected as their expression by preimplantation stages of embryonic development has not yet been described; *NEU2* and *PAI1* were chosen due to their potential developmental significance. No staining could be observed when the primary antibody was omitted and replaced with serum block solution or normal serum obtained from the species in which the primary antibody was raised. Signal for all five proteins was detected and localized to trophoblast cells. FGB and NEU2 showed strong staining intensity, and FGA and FGB showed moderately strong staining intensity, while PAI1 exhibited moderate staining intensity. In the case of FGA, FGB, and FGG, the acellular capsule also showed a strong staining intensity. NEU2 resulted in faint staining of capsular material, while PAI1 did not result in any visible staining signal associated with the capsule.

Western Blotting

A similar pattern of immunoreactivity was observed for FGA and FGG. Immunoreactive bands at the respective

TABLE 2. Transcripts showing differential mRNA expression levels in equine conceptuses of different developmental stages.

Equine gene/cDNA or homologue	Gene symbol	GenBank accession no.	Putative function	Day 10[a]	Day 12[a]	Day 14[a]
E. caballus similar to apolipoprotein B-100	*APOB*	XM_001501679	Lipid metabolic process; signal transduction; cholesterol transport	22.1	77.4	142.0
E. caballus clone CH241-268N6	–	AC159151	–	46.0	112.6	127.6
E. caballus similar to Fibrinogen beta chain precursor	*FGB*	XM_001500955	Positive regulation of cell proliferation; signal transduction	17.9	91.9	107.0
Plasmodium vivax hypothetical protein	_	XM_001616478	–	44.4	136.3	68.7
E. caballus oncostatin M receptor	*OSMR*	XM_001496993	Cell surface receptor linked signal transduction; cell proliferation	33.7	60.6	68.5
H. sapiens chromosome 17, clone RP11-106A7	–	AC126391	–	48.4	76.8	66.0
Mus musculus BAC clone RP23-451M13	–	AC125352	–	49.4	117.7	62.3
Uncultured bacterium zdt-14e13 clone zdt-14e13	–	AC150249	–	34.9	113.6	60.2
Oryza sativa (japonica cultivar-group) genomic DNA	–	AP008211	–	26.2	48.0	57.0
E. caballus similar to receptor activity-modifying protein 1	*RAMP1*	XM_001496783	G-protein coupled receptor protein signaling pathway	36.0	45.6	54.8
H. sapiens single-stranded DNA binding protein 2	*SSBP2*	NM_012446	–	4.6	26.8	50.3
Tetrahymena thermophila SB210 zinc finger domain, LSD1 subclass family protein	–	XM_001012543	–	24.0	62.6	40.6
H. sapiens ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	*ST6GALNAC3*	NM_001160011	–	36.9	39.1	37.1
E. caballus dystrophin related protein 2	*DRP2*	XM_001493019	Calcium ion binding; protein binding	13.2	40.6	35.1
E. caballus similar to granzyme B	*GZMB*	XM_001488193	Apoptosis; proteolysis	3.7	26.5	33.4
Canis familiaris coagulation factor X	*F10*	XM_534191	Blood coagulation; proteolysis; calcium ion binding	27.5	262.9	32.7
H. sapiens ATG3 autophagy related 3 H.log	*ATG3*	NM_022488	–	36.4	31.5	26.6
E. caballus similar to interleukin 17F	*IL17F*	XM_001498910	Negative regulation of angiogenesis; regulation of IL8 biosynthetic process	14.2	91.7	25.0
E. caballus eprorelaxin	*RLN*	NM_001081809	Signal transduction; hormone activity	7.7	30.2	23.7
H. sapiens chromosome 18, clone RP11-640A1	–	AC100843	–	6.8	13.1	18.5
E. caballus similar to Apolipoprotein C-II precursor	*APOC2*	XM_001502376	Lipid transport; lipoprotein lipase activity	7.4	33.5	18.1
E. caballus similar to tetratricopeptide repeat domain 36	*TTC36*	XM_001500960	Binding	21.4	57.8	18.1
E. caballus similar to echinoderm microtubule associated protein like 5	*EML5*	XM_001495712	–	9.0	12.2	16.3
H. sapiens fibroblast growth factor 13	*FGF13*	NM_001139498	Cell-cell signaling; growth factor	2.4	12.9	16.2
E. caballus similar to SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a1	*SMARCA1*	XM_001500518	Transcription; ATP-dependent chromatin remodeling	3.9	9.6	15.4
Mus musculus lecithin-retinol acyltransferase (phosphatidylcholine-retinol-O-acyltransferase)	*Lrat*	NM_023624	Vitamin A metabolic process; transferase activity	10.9	26.2	15.3
E. caballus similar to odd-skipped related 2 (*Drosophila*)	*OSR2*	XM_001491780	Positive regulation of cell proliferation; nucleic acid binding; metal ion binding	5.1	9.4	14.6
E. caballus similar to Zinc finger CCCH type antiviral protein 1	*ZC3HAV1*	XM_001499163	Nucleic acid binding; metal ion binding; NAD+ ADP-ribosyltransferase activity	10.9	15.4	13.9
H. sapiens secreted protein, acidic, cysteine-rich (osteonectin)	*SPARC*	NM_003118	Transmembrane receptor protein tyrosine kinase signaling; collagen binding	4.6	13.6	13.5
E. caballus similar to COL4A1 protein	*COL4A1*	XM_001496530	Phosphate transport; extracellular matrix structural constituent	3.7	9.5	13.2

TABLE 2. *Continued.*

Equine gene/cDNA or homologue	Gene symbol	GenBank accession no.	Putative function	Day 10[a]	Day 12[a]	Day 14[a]
H. sapiens solute carrier family 39 (zinc transporter), member 2	*SLC39A2*	NM_014579	Ion transport	4.3	9.7	12.6
H. sapiens mitogen-activated protein kinase kinase kinase 8	*MAP3K8*	NM_005204	Protein amino acid phosphorylation; nucleotide binding	3.1	3.4	11.9
H. sapiens guanine deaminase	*GDA*	NM_004293	Nucleic acid metabolic process	4.2	12.4	11.8
Bos taurus similar to CCCH-type zinc finger antiviral protein	*ZC3HAV1*	XM_866143	Nucleic acid binding; metal ion binding; NAD+ ADP-ribosyltransferase activity	10.9	18.8	11.7
Sus scrofa CD3e molecule, epsilon (CD3-TCR complex)	*CD3E*	NM_214227	Cell surface receptor linked signal transduction; T cell receptor signaling	2.7	6.9	10.8
Horse interferon-alpha-1 gene	*IFNA1*	NM_001099441	Hematopoietin/interferon-class (D200-domain) cytokine receptor binding	4.0	8.3	10.7
E. caballus mRNA for aquaporin 5	*AQP5*	NM_001114607	Water transport	4.6	9.5	10.7
H. sapiens cDNA FLJ37631 fis, clone BRCOC2015944	–	AK094950	–	4.0	10.0	10.0
H. sapiens sodium channel, nonvoltage-gated 1, delta	*SCNN1D*	NM_001130413	Amiloride-sensitive sodium channel activity	4.2	22.7	9.8
Phosphorylase, glycogen, liver	*PYGL*	XM_001496917	Phosphorylase activity; ttransferring glycosyl groups; nucleotide binding	6.1	21.0	9.5
E. caballus similar to WAP four-disulfide core domain 3	–	XM_001500769	Serine-type endopeptidase inhibitor activity	4.4	9.2	9.4
H. sapiens cadherin 16, KSP-cadherin	*CDH16*	XM_546890	Homophilic cell adhesion	4.4	12.5	9.2
LOC100067095 similar to Aminoadipate aminotransferase	*AADAT*	XM_001498966	2-aminoadipate transaminase activity	4.4	8.0	9.1
E. caballus similar to B-cell translocation protein 2	*BCL2*	XM_001488564	Transcription factor activity; negative regulation of apoptosis	2.3	5.2	8.7
H. sapiens oxoglutarate dehydrogenase-like, nuclear gene encoding mitochondrial protein	*OGDHL*	XM_001500169	Glycolysis	3.8	15.2	8.7
E. caballus solute carrier family 2 (facilitated glucose/fructose transporter), member 5	*SLC2A5*	NM_001081877	Sugar:hydrogen ion symporter activity	3.0	8.0	8.6
E. caballus similar to Embigin H.log (mouse)	*EMB*	XM_001497895	Integral to membrane	3.1	6.0	8.6
E. caballus similar to zona pellucida B	*ZP4*	XM_001490753	Receptor activity	5.1	8.1	8.6
H. sapiens myosin, light chain 3, alkali; ventricular, skeletal, slow	*MYL3*	NM_000258	Muscle contraction; calcium ion binding	3.8	7.4	8.1
E. caballus similar to phospholipase A2, group III	*PLA2G3*	XM_001497343	Phospholipid metabolic process; calcium ion binding	3.9	17.1	7.8
E. caballus similar to Putative phospholipase B-like 1 precursor (Lamina ancestor H.log 1)	*PLBD1*	XM_001497121	Extracellular region	2.2	6.3	7.6
E. caballus similar to neuromedin B	*NMB*	XM_001502607	Cell-cell signaling; hormone activity	4.2	7.2	7.6
SH3-domain kinase binding protein 1	*SH3KBP1*	XM_001917276	Cell-cell signaling; apoptosis	2.7	5.8	7.5
H. sapiens chromosome 5 open reading frame 13	*C5orf13*	NM_001142483	Regulation of transforming growth factor beta receptor signaling pathway	3.7	5.5	7.5
H. sapiens solute carrier family 38, member 1	*SLC38A1*	XM_001094126	Neutral amino acid, sodium ion transport	4.2	9.6	7.5
H. sapiens POC1 centriolar protein H.log B	*POC1B*	NM_172240	–	4.9	14.1	7.3
E. caballus desmocollin 2	*DSC2*	XM_001496306	Cell adhesion; calcium ion binding	3.8	10.2	7.2
E. caballus similar to zinc binding alcohol dehydrogenase, domain containing 2	*ZADH2*	XM_001493715	Oxidoreductase activity; zinc ion binding	2.1	6.1	7.1
H. sapiens chromosome 8, clone RP11-16M8	–	AC012349	Cell division	2.6	3.9	7.0
E. caballus similar to latent transforming growth factor beta binding protein 1	*LTBP1*	XM_001500182	Growth factor binding; calcium ion binding	4.6	12.0	6.9

TABLE 2. *Continued.*

Equine gene/cDNA or homologue	Gene symbol	GenBank accession no.	Putative function	Day 10[a]	Day 12[a]	Day 14[a]
E. caballus similar to C1orf88 protein	–	XM_001494485	–	6.0	6.2	6.9
E. caballus hypothetical protein LOC100066131	–	XM_001501228	–	3.5	11.2	6.8
PREDICTED: *Bos taurus* CD24 antigen-like	*CD24*	XM_002685670	Positive regulation of activated t cell proliferation; cell migration	7.1	8.3	6.8
E. caballus hypothetical LOC100050360	–	XM_001489098	–	3.1	7.8	6.8
H. sapiens fibronectin leucine rich transmembrane protein 2	*FLRT2*	XM_510103	Cell adhesion; lipid catabolic process; phospholipase A2 activity	3.6	8.2	6.7
E. caballus phosphatase and actin regulator 3	*PHACTR3*	XM_001491744	Protein phosphatase inhibitor activity; actin binding	4.0	6.2	6.6
E. caballus similar to Vitamin K-dependent protein S	*PROS1*	XM_001502990	Blood coagulation; calcium ion binding	2.6	4.3	6.6
E. caballus interleukin 18	*IL18*	NM_001082512	Immune response; interleukin-1 receptor binding	2.6	5.9	6.5
H. sapiens chitinase 3-like 2	*CHI3L2*	NM_004000	Carbohydrate metabolic process	4.5	5.4	6.1
E. caballus similar to olfactory receptor Olr1012	–	XM_001489337	–	4.6	7.1	5.9
LOC100051297 similar to THRSP protein	*THRSP*	XM_001493089	Lipid metabolic process; transcription	4.6	8.2	5.8
E. caballus monoamine oxidase B (MAOB), nuclear gene encoding mitochondrial protein	*MAOB*	NM_001081833	Oxidoreductase activity; electron carrier activity	5.8	6.5	5.7
E. caballus Bardet-Biedl syndrome 7	*BBS7*	XM_001503097	Response to stimulus	2.1	3.4	5.5
E. caballus similar to apolipoprotein M	*APOM*	XM_001490422	Lipid transporter activity	2.6	4.3	5.5
Branched chain keto acid dehydrogenase E1, beta polypeptide	*BCKDHB*	XM_532213	Carboxy-lyase activity	2.7	3.3	5.2
E. caballus plasminogen activator inhibitor-1	*PAI1*	XM_001492517	Regulation of angiogenesis; serine-type endopeptidase inhibitor activity	2.7	4.4	5.0
Bos taurus similar to hCG2036558	–	XM_001256327	–	2.1	4.2	4.9
H. sapiens phosphorylase kinase, gamma 1 (muscle)	*PHKG1*	NM_006213	Protein amino acid phosphorylation	2.7	3.9	4.8
H. sapiens malignant fibrous histiocytoma amplified sequence 1	*MFHAS1*	NM_004225	Small GTPase mediated signal transduction	3.9	4.0	4.8
E. caballus carbohydrate (N-acetylgalactosamine 4–0) sulfotransferase 8	*CHST8*	XM_001489047	Hormone biosynthetic process; carbohydrate metabolic process	2.8	6.7	4.6
Bos taurus spondin 1, extracellular matrix protein	*SPON1*	NM_174743	Cell adhesion	2.7	3.2	4.6
E. caballus annexin A2	*ANXA2*	NM_001123380	Collagen fibril organization; phospholipase inhibitor activity	2.3	3.4	4.6
E. caballus suppression of tumorigenicity 7 like	*ST7L*	XM_001498901	–	2.0	3.6	4.6
E. caballus similar to leucine rich repeat containing 56	*LRRC56*	XM_001489689	Protein binding	2.7	12.4	4.6
E. caballus similar to methylmalonate-semialdehyde dehydrogenase	*ALDH6A1*	XM_001490403	Pyrimidine nucleotide metabolic process	2.1	3.1	4.4
PREDICTED: *E. caballus* similar to PPAR gamma coactivator-1	*PPAR*	XM_001499929	Positive regulation of histone acetylation; transcription	2.4	2.5	4.4
E. caballus sodium/potassium dependent ATPase beta-1 subunit	*ATP1B1*	XM_001491201	Ion transport	2.7	7.5	4.4
E. caballus gasdermin-like	*GSDML*	XM_001497891	Catagen	3.3	7.3	4.2
E. caballus tripartite motif-containing 2	*TRIM2*	XM_001501173	Metal ion binding; protein binding	3.1	4.3	4.2
E. caballus similar to Transcobalamin II; macrocytic anemia	*TCN2*	XM_001494787	Ion transport	2.4	5.1	4.1
H. sapiens inositol(myo)-1(or 4)-monophosphatase 2	*IMPA2*	NM_014214	Signal transduction; phosphate metabolic process	2.7	3.2	4.1
E. caballus N-acetylated alpha-linked acidic dipeptidase-like 2	*NAALADL2*	XM_001495653	Carbohydrate metabolic process	2.1	2.5	3.9

TABLE 2. *Continued.*

Equine gene/cDNA or homologue	Gene symbol	GenBank accession no.	Putative function	Day 10[a]	Day 12[a]	Day 14[a]
H. sapiens insulin-like growth factor 2 mRNA binding protein 2	*IGF2BP2*	NM_001007225	–	2.2	3.9	3.9
H. sapiens integrin, alpha 6	*ITGA6*	NM_000210	Cell adhesion; integrin-mediated signaling	2.3	2.4	3.8
Bos taurus histone cluster 2, H2be	*HIST2H2BE*	NM_001099384	–	2.2	4.8	3.8
E. caballus similar to fucosyltransferase 8 (alpha (1,6) fucosyltransferase)	*FUT8*	XM_001499525	Cell migration; transforming growth factor beta receptor signaling pathway	2.5	3.1	3.8
E. caballus similar to tyrosine protein-kinase STYK1 (Serine/threonine/tyrosine kinase 1) (Novel oncogene with kinase domain)	*STYK1*	XM_001500326	Protein amino acid phosphorylation; non-membrane spanning protein tyrosine kinase activity	4.5	4.3	3.7
Bos taurus transmembrane protein 2	*TMEM2*	NM_001192415	–	2.3	2.7	3.7
E. caballus prostaglandin-endoperoxide synthase 1	*PTGS1*	XM_001501406	Prostaglandin biosynthetic process	3.4	8.3	3.7
H. sapiens tetraspanin 12	*TSPAN12*	NM_012338	Integral to membrane	2.3	2.4	3.6
PREDICTED: *E. caballus* similar to phospholipase A2, group VII	*PLA2G7*	XM_001502666	Lipid catabolic process; inflammatory response	2.2	4.1	3.6
Pan troglodytes desmoglein 2	*DSG2*	XM_512079	Homophilic cell adhesion	2.5	2.2	3.2
E. caballus similar to ATPase, H+ transporting, lysosomal accessory protein 2	*ATP6AP2*	XM_001489026	Receptor activity	2.1	3.5	3.2
E. caballus nidogen 1	*NID1*	XM_001916503	Cell adhesion; calcium ion binding	2.6	2.8	3.1
E. caballus bobby sox H.log (*Drosophila*)	*BBX*	XM_001503327	Regulation of transcription, DNA-dependent	2.0	2.9	3.0
E. caballus similar to cytochrome P450, family 2, subfamily F, polypeptide 1	*CYP2F1*	XM_001498170	Electron transport	2.2	4.0	3.0
H. sapiens NIPA-like domain containing 2	*NIPAL2*	NM_024759	–	2.6	2.3	2.9
E. caballus similar to Abhydrolase domain-containing protein 6	*ABHD6*	XM_001489265	Hydrolase activity	2.2	3.2	2.8
E. caballus similar to CD82 antigen	*CD82*	XM_001489923	Protein binding	2.4	3.8	2.8
H. sapiens cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	*CDKN2C*	NM_001262	Negative regulation of cell growth	2.0	2.6	2.7
E. caballus similar to FAM59A protein	*FAM59A*	XM_001496712	Structural molecule activity	2.2	2.4	2.6
H. sapiens fibronectin type III and SPRY domain containing 1-like	*FSD1L*	NM_001145313	–	2.3	2.7	2.6
E. caballus clone CH241-67M21	–	AC124908	–	2.0	7.5	2.5
E. caballus similar to melanoma antigen, family D 1	*MAGED1*	XM_001914933	Regulation of apoptosis; negative regulation of epithelial cell proliferation	2.0	2.3	2.5
Sus scrofa similar to pyruvate dehydrogenase phosphatase	–	XM_001924838	Protein amino acid dephosphorylation; calcium ion binding	2.9	2.6	2.5
E. caballus S100 calcium binding protein A10	*S100A10*	NM_001163867	Signal transduction	2.2	3.5	2.5
H. sapiens chromosome 15, clone RP11-15F13	–	AC011648	–	2.1	2.7	2.2
E. caballus similar to C-1-tetrahydrofolate synthase, cytoplasmic (C1-THF synthase)	*MTHFD1*	XM_001499131	Amino acid biosynthetic process	0.3	0.5	0.4
E. caballus similar to caudal type homeobox 1	*CDX1*	XM_001501485	Transcription factor activity	0.3	0.2	0.4
E. caballus similar to Keratin, type I cytoskeletal 14 (Cytokeratin-14) (CK-14) (Keratin-14) (K14)	*CK14*	XM_001496937	Epithelial cell differentiation	0.4	0.3	0.4
H. sapiens hydroxysteroid (17-beta) dehydrogenase 14	*HSD17B14*	XM_001489431	Steroid metabolic process	0.5	0.4	0.4
Canis Familiaris chromosome 20, clone XX-21B12	_	AC187030	Protein binding	2.6	2.9	0.4
E. caballus similar to Nedd4 binding protein 3	*N4BP3*	XM_001502111	_	0.5	0.4	0.4
E. caballus similar to GRAM domain-containing protein 1A	*GRAMD1A*	XM_001491659	Integral to membrane	0.4	0.3	0.3

TABLE 2. *Continued.*

Equine gene/cDNA or homologue	Gene symbol	GenBank accession no.	Putative function	Day 10[a]	Day 12[a]	Day 14[a]
H. sapiens BARX homeobox 1	*BARX1*	NM_021570	Cell-cell signaling; epithelial cell differentiation; transcription	0.3	0.2	0.3
E. caballus similar to Rho-related BTB domain containing 3	*RHOBTB3*	XM_001503702	GTPase activity	0.3	0.2	0.3
E. caballus similar to arylformamidase	*AFMID*	XM_001491048	Tryptophan catabolic process	0.5	0.2	0.3
E. caballus CD44 molecule (Indian blood group)	*CD44*	NM_001085435	Cell adhesion	0.4	0.2	0.3
E. caballus carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	*CAD*	XM_001502425	Glutamine metabolic process	0.4	0.2	0.2
Bos taurus 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	*PFKFB3*	NM_001077837	Kinase activity	0.4	0.2	0.2
E. caballus similar to zinc finger protein 342	*ZNF296*	XM_001502445	Transcription	0.3	0.2	0.2
E. caballus similar to limbin	*EVC2*	XM_001499672	Transport	0.3	0.4	0.2
E. caballus similar to tripartite motif-containing 47	*TRIM47*	XM_001491966	Metal ion binding	0.4	0.1	0.2
E. caballus similar to CCAAT/enhancer binding protein epsilon	*CEBPE*	XM_001493841	Transcription; macrophage differentiation	0.4	0.2	0.2
H. sapiens ADAM metallopeptidase with thrombospondin type 1 motif, 5	*ADAMTS5*	XM_001103439	Metalloendopeptidase activity	0.2	0.04	0.2
Canis familiaris similar to G1/S-specific cyclin D2	*CCND2*	XM_849493	Cell division	0.2	0.1	0.2
H. sapiens DNA cytosine-5 methyltransferase 3 beta 1	*DNMT3B*	NM_006892	DNA methylation	0.3	0.1	0.2
E. caballus similar to transmembrane 7 superfamily member 3	*TM7SF3*	XM_001502786	Integral to membrane	0.4	0.3	0.2
E. caballus similar to solute carrier family 5 (sodium/glucose cotransporter), member 11	*SLC5A11*	XM_001501307	Ion transport; carbohydrate transport	0.2	0.1	0.1
E. caballus similar to uroplakin III	*UPK3A*	XM_001488250	Protein binding	0.3	0.1	0.1
E. caballus sortilin-related receptor, L(DLR class) A repeats-containing	*SORL1*	XM_001501368	Steroid metabolic process	0.4	0.1	0.1
E. caballus B-cell CLL/lymphoma 6 (zinc finger protein 51)	*BCL6*	XM_001499782	Transcription; protein localization	0.4	0.1	0.1
Macaca mulatta similar to formin-like 2	*FMNL2*	XM_001084144	Actin cytoskeleton organization; transcription	0.3	0.2	0.1
E. caballus similar to matrilin 4	*MATN4*	XM_001500576	Extracellular region	0.2	0.3	0.1
H. sapiens ring finger protein 125	*RNF125*	NM_017831	Ubiquitin cycle	0.3	0.1	0.1
E. caballus similar to phospholipase A2 precursor (phosphatidylcholine 2-acylhydrolase) (group IB phospholipase A2)	*PLA2G1B*	XM_001489253	Phospholipid metabolic process; actin filament organization; signal transduction	0.1	0.03	0.1
E. caballus similar to collagen, type XXVIII	*COL28A1*	XM_001494969	Serine-type endopeptidase inhibitor activity	0.3	0.0	0.05
H. sapiens nuclear receptor interacting protein 3	*NRIP3*	XM_001504910	Proteolysis	0.3	0.0	0.04
H. sapiens spondin 2, extracellular matrix protein	*SPON2*	NM_001128325	Cell adhesion	0.3	0.0	0.03
E. caballus solute carrier family 26, member 4	*SLC26A4*	XM_001491415	Transport	0.4	0.1	0.03
H. sapiens ankylosis, progressive H.log (mouse)	*ANKH*	NM_054027	–	0.2	0.1	0.03

[a] Expression level in Day-10, -12, and -14 conceptuses, expressed as fold change relative to Day-8 conceptuses.

molecular weight of FGA (60 kDa) and FGG (57 kDa) were observed for Day-14 and Day-12 conceptuses, yolk sac fluid, CCM, and capsular material. No signal could be observed in Day-8 and Day-10 embryos. Using the anti-FGA antibody, in all samples except Day-8 conceptus, a faint higher molecular weight band could be observed at 250 kDa (Fig. 5).

DISCUSSION

Successful establishment of pregnancy is characterized by temporally and spatially regulated events in the endometrium and conceptus. The pregnancy recognition signal in the mare has eluded identification thus far, and limited information is available about transcriptional activity of preimplantation

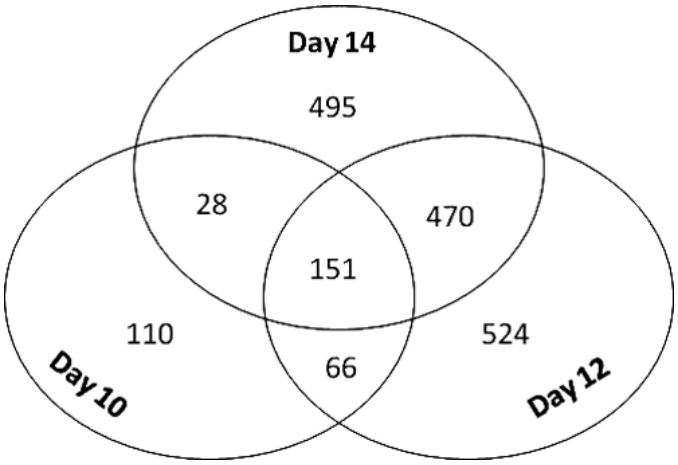

FIG. 1. Venn diagram depicting the number of differentially expressed transcripts in Day-10, -12, and -14 conceptuses when compared to Day-8 conceptuses. Numbers inside each compartment represent the number of transcripts that are differentially expressed in only one developmental stage or that overlap between two or three developmental stages, respectively.

equine conceptuses. The objective of the present study was to identify differences in gene expression between Day-8, -10, -12, and -14 equine conceptuses with Day-8 conceptuses representing a stage before MRP, and the remaining developmental stages coinciding with MRP. Conceptuses were retrieved on the respective days after ovulation and subjected to holistic gene expression analysis (i.e., microarray analysis). A custom-designed whole-genome microarray based on publicly available sequences supplemented with expression data obtained by high-throughput sequencing was used [13]. Day-8 conceptuses were paired with Day-10, -12, and -14 conceptuses for array analysis. Four conceptuses per time point were analyzed. Totals of 355, 1211, and 1144 transcripts were differentially expressed between Day-8 and Day-10, -12, and -14 conceptuses, respectively. The main criterion for differential expression was a difference in signal intensity of 2.0-fold or more in all four conceptuses included in the study, a ''well above background'' call, and a P value of 0.01 or less. Quantitative PCR on a subset of selected genes confirmed expression data obtained by microarray, supporting the results. Protein expression of selected transcripts was confirmed using Western blotting and IHC. GO classification of up-regulated genes revealed that the majority of transcripts with higher expression in Day-10, -12, and -14 conceptuses when compared to Day-8 conceptuses localize to the extracellular region and/or are intrinsic to the plasma membrane. This reflects the embryo-maternal cross-talk, as gene products released into the conceptus' surroundings can act upon the uterine environment to elicit changes necessary for establishment of pregnancy. Significantly enriched transcripts for the GO category ''Biological Process'' reflect the response of the developing conceptus to various stimuli, foremost hormone and extracellular stimuli. This mirrors the response to factors released by the endometrium during early pregnancy, as well as the response to steroid hormones secreted by the equine conceptus itself. The enhanced expression of genes involved in cholesterol transport reflects the active synthesis of steroid hormones. The biological processes ''lipid biosynthetic

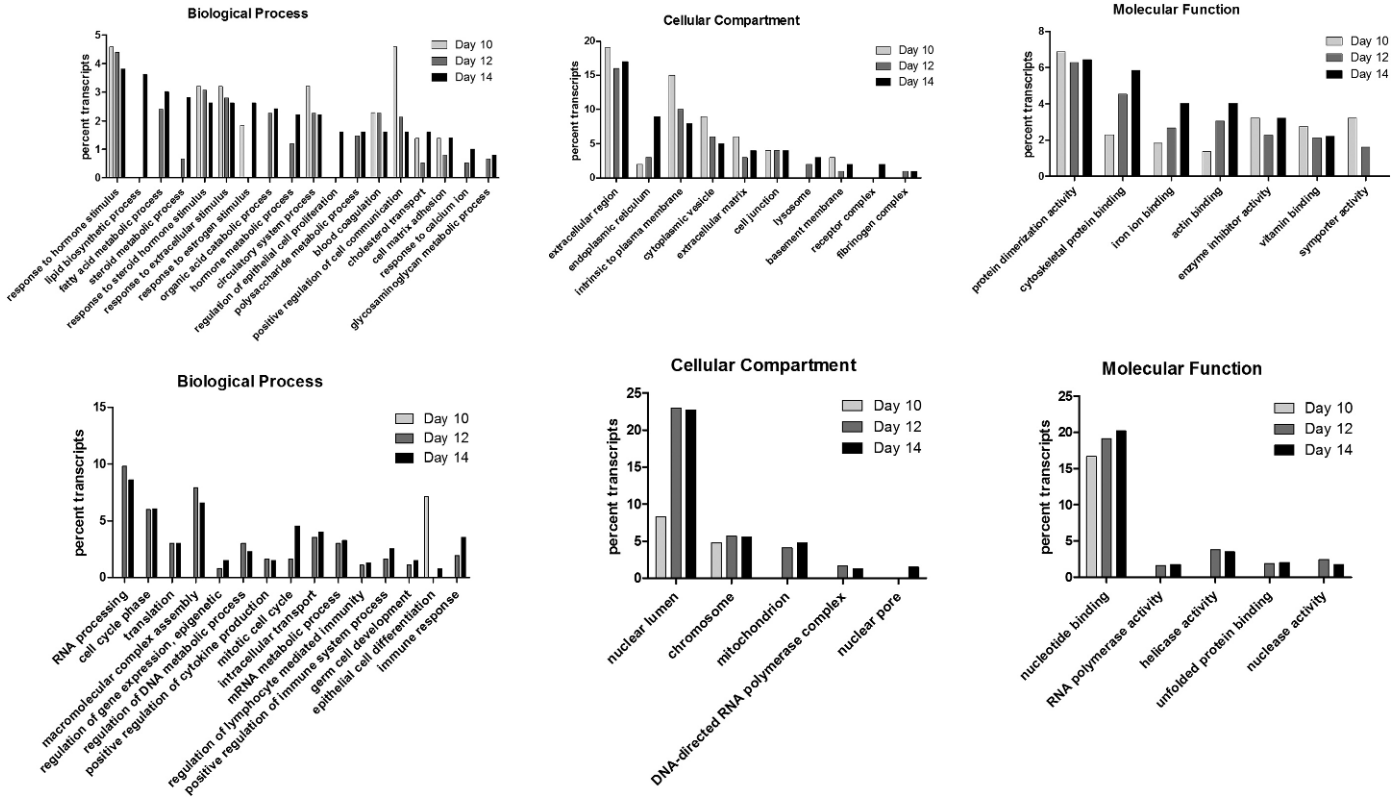

FIG. 2. Classification into functional categories of genes differentially regulated in Day-10, -12, and -14 embryos when compared to Day-8 embryos. Upper row, up-regulated transcripts; lower row, down-regulated transcripts. GO processes are ranked by the percentage of genes falling in to the respective category. Genes may appear multiple times within these hierarchies so that the number of genes provided in this figure is relative to a certain biological process, and is not absolute.

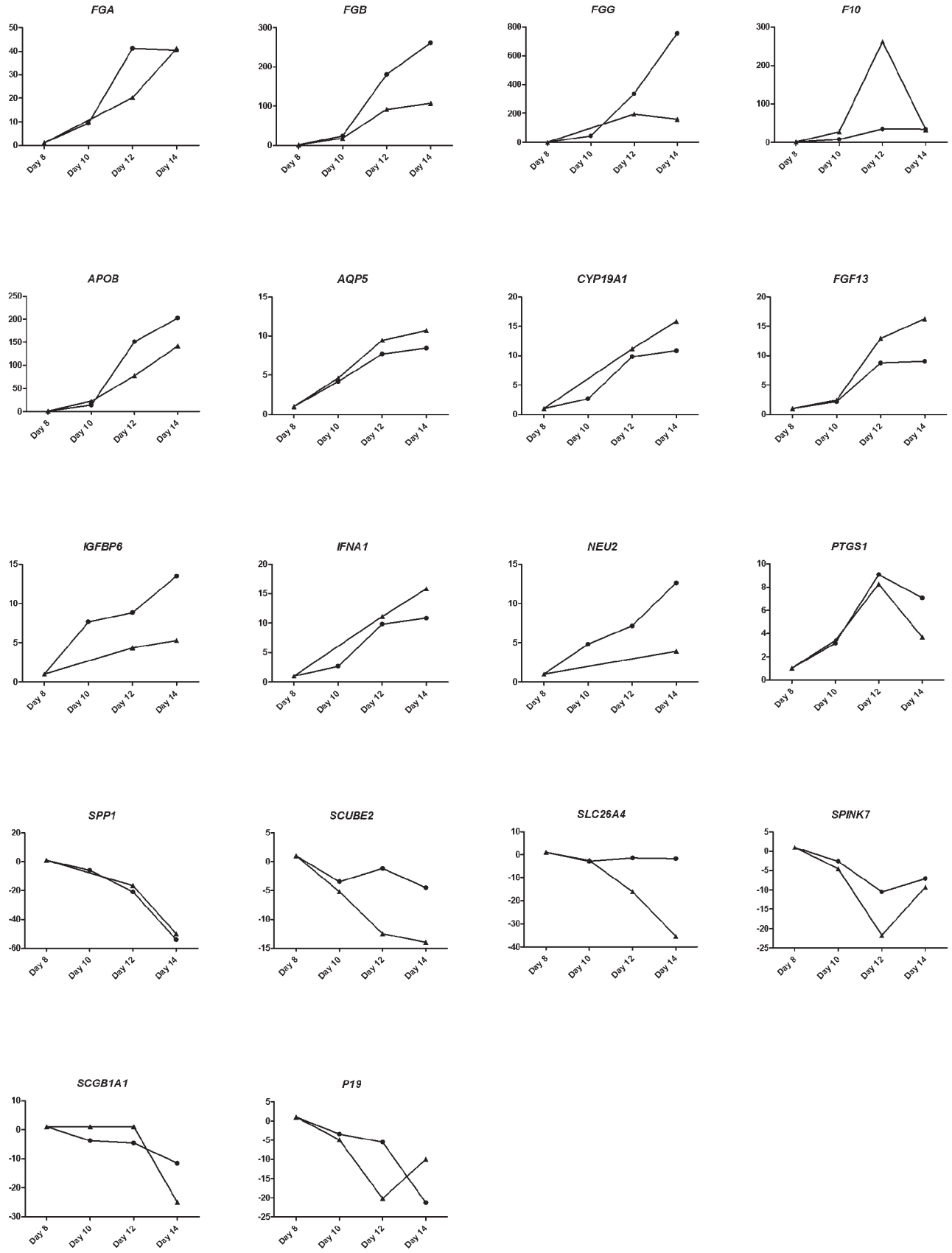

FIG. 3. Validation of microarray results by RT-qPCR on a subset of up- and down-regulated transcripts. Line with dot, fold change obtained by RT-qPCR; line with triangle, fold change obtained by microarray analysis.

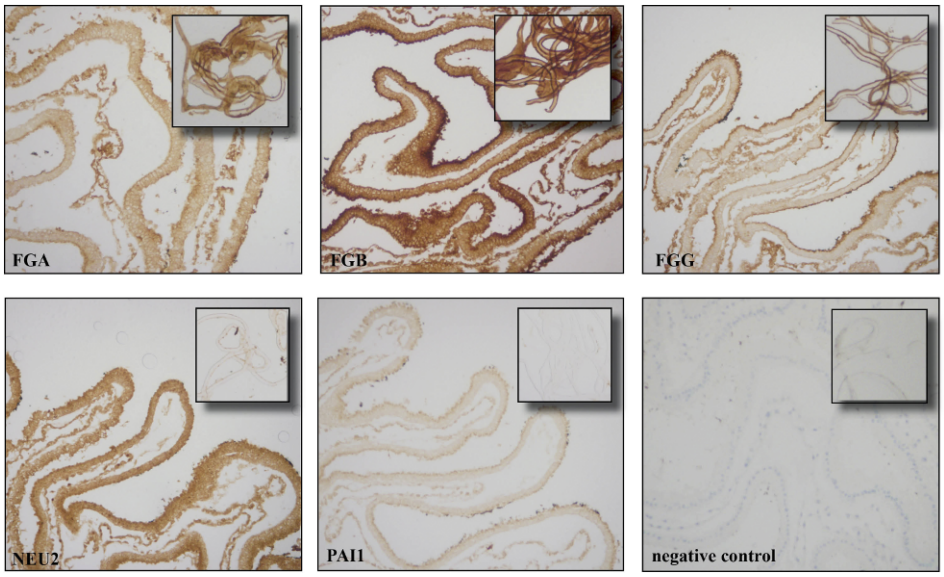

FIG. 4. Immunohistochemistry. Conceptuses were retrieved 14 days after ovulation for the detection of FGA, GFB, FGG, NEU2, and PAI1. Inserted images show capsular material. All images are shown at 200× magnification.

process'' and ''fatty acid metabolic process'' depict the increased energy need of the conceptus and the production of prostaglandins. Down-regulated transcripts were significantly enriched for the cellular compartment ''nucleus,'' which is in accordance with the biological process in which these transcripts are involved, the regulation of gene expression. The need to protect the semiallograft conceptus from the uterine immune system can be seen in that the down-regulated transcripts are significantly enriched for genes in the positive regulation of the immune system.

A subset of genes identified as differentially expressed, such as *BRCA1*, has previously been described in the context of early pregnancy in species other than the horse. *BRCA1*, best known for its function as tumor suppressor gene, showed higher expression in Day-12 and -14 conceptuses. A knockout model for BRCA1 in mice has demonstrated a critical role for this gene in early embryonic development [16], and recently BRCA1 has been suggested as a marker of implantation-competent blastocysts in the mouse [17]. Given the expression in equine conceptuses, it seems that the expression of *BRCA1* is required for successful embryonic development across species. Other transcripts, such as *FGF2*, showed a different expression pattern than observed previously in other species. *FGF2* showed a decrease in expression in the current study as conceptus development proceeded, while *FGF2* mRNA abundance temporarily increases in cattle, coinciding with onset of elongation of the conceptus [18]. Unlike in cattle, increased expression of FGF2 does not seem to be critical for proper embryonic development in the horse; however, one has to keep in mind that equine conceptuses do not undergo elongation, and that FGF2 may be important for conceptus' implantation, which occurs at a later stage in equine pregnancy. Besides transcripts previously described as expressed by early stages of embryonic development in species other than the horse, a set of genes was identified not yet known to be expressed by embryonic tissue. In the following, the discussion highlights the potential function of selected transcripts apparently unique to equine conceptuses.

Fibrinogen Alpha, Beta, and Gamma

Fibrinogen is a soluble plasma glycoprotein synthesized by the liver that is converted by thrombin into fibrin during blood coagulation. Fibrinogen occurs as a dimer, each monomer being composed of three nonidentical chains—alpha, beta and gamma—linked together by several disulphide bonds [19]. A surprising finding in the current study was the up-regulation of all three fibrinogen subunits, alpha, beta, and gamma. Microarray data were confirmed using qPCR; all three subunits showed a similar expression pattern, with low mRNA levels observed in Day-8 embryos that gradually increase in Day-10, -12, and -14 conceptuses. Fibrinogen gamma showed the largest

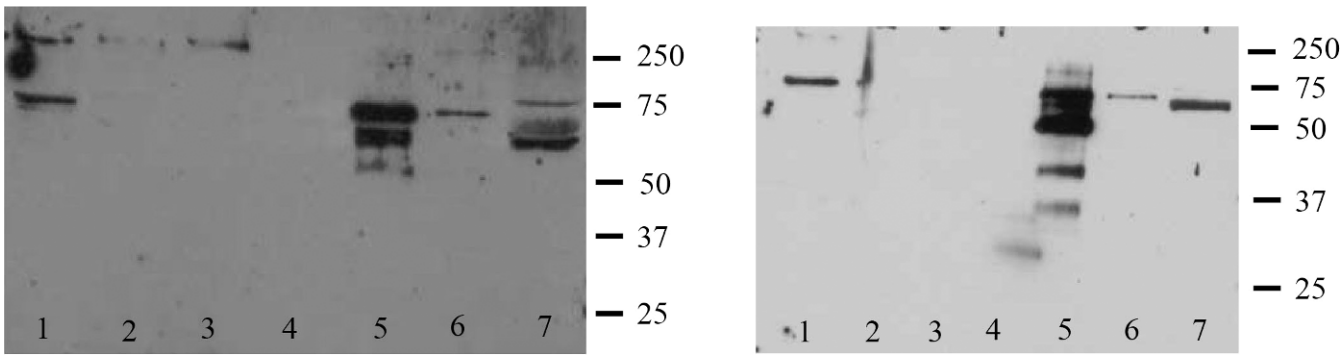

FIG. 5. Western Blotting. Protein was isolated from Day-14, -12, -10, and -8 conceptuses, capsule, yolk sac, and conceptus-conditioned medium for Western Blot analysis using anti-human FGA and FGG antibodies. Left, FGA blot; right, FGG blot. 1, Day-14 conceptus; 2, Day-12 conceptus; 3, Day-10 conceptus; 4, Day-8 conceptus; 5, capsule; 6, CCM; 7: yolk sac.

fold-change increase, followed by fibrinogen beta and alpha. Western blot analysis on conceptus tissue, capsule, yolk sac, and CCM confirmed the presence of fibrinogen alpha and gamma protein in conceptus tissue, its association with capsular material, its presence in yolk sac fluid, and the secretion of the respective proteins into the surroundings (i.e., CCM of the conceptus). It is therefore likely that fibrinogen chains of conceptus' origin are released into the uterine lumen during early pregnancy and can act upon the endometrium. During the Western blot analysis, a higher molecular weight band (approximately 250 kDa) was observed. It is possible that this higher molecular weight band represents intact fibrinogen and is detected due to cross-reactivity of the subunit-specific antibodies with intact fibrinogen. In humans, maternal fibrinogen is required to support pregnancies by maintaining hemostatic balance and stabilizing uteroplacental attachment at the fibrinoid layer found at the fetal-maternal junction [20]; likewise, during embryonic development in the mouse, fibrinogen stabilizes placental-maternal attachment [21]. This is the first reported observation of expression of fibrinogen in preimplantation embryos. The production of fibrinogen subunits and their likely secretion into the uterine environment is a puzzling finding, given the role of fibrinogen in inflammatory processes. Soluble fibrinogen has been shown to activate neutrophils, resulting in degranulation, enhancement of phagocytosis, and apoptosis delay [22]. Fibrinogen is also able to stimulate chemokine production by macrophages [23]. In the context of early pregnancy and the need for a tightly controlled endometrial immune system, to avoid rejection of the semiallograft conceptus, it seems unlikely that fibrinogen exerts the above-mentioned actions. At this time, the biological implication of fibrinogen alpha, beta, and gamma at the conceptus-maternal interface in the horse remains unclear and warrants further investigation. A role for fibrinogen in the inhibition of luteolysis seems unlikely, given that the molecular weight of the pregnancy recognition signal secreted by the equine conceptus lies between 1000 and 10′000 Da and is not altered by proteinase K treatment [24].

Genes Related to the Equine Capsule

The equine conceptus is surrounded by an acellular glycoprotein capsule, a structure unique amongst domestic animals, with the glycoproteins being derived from the trophoblast [25]. This capsule contains glycoproteins resembling those of the mucin family, with sialic acid making up a high content of the carbohydrates [26]. The sialylation of glycoproteins takes place in the Golgi apparatus [26, 27], and different sialyltransferases catalyze the transfer of sialic acid residues onto glycan structures [28]. The up-regulated transcript *SLC35A1*, also known as CMP-sialic acid transporter, transports CMP-sialic acid into the lumen of the Golgi complex, supplying substrate for the posttranslational modification of proteins [29]. Sialyltransferases are enzymes that transfer sialic acid to nascent oligosaccharides, each exhibiting specificity for a particular sugar substrate [28]. Four sialyltransferases, *ST6GALNAC3*, *ST6GAL1*, *ST8SIA4*, and *ST8SIA2*, showed elevated mRNA expression levels in older conceptuses. The up-regulation of *SLC35A1* and the four sialyltransferases in the current study is hypothesized to reflect the biosynthetic activity of the equine conceptus with regard to production of capsular material. Moreover, the composition of the capsule is not static, but rather changes as conceptus development proceeds. So does the sialic acid content decrease markedly from about Day 16, coinciding with the conceptus' fixation in the uterus [30]. The gene *NEU2* encodes for an enzyme known as neuraminidase 2 or sialidase 2. This gene belongs to a family of glycohydrolytic enzymes, which cleave sialic acid residues from the oligosaccharide components of glycoconjugates [31]. Expression studies in COS7 cells confirmed that this gene encodes a functional sialidase. Its cytosolic localization was demonstrated by cell fractionation experiments [32]. Arar and coworkers [33] demonstrated that the capsule of prefixation conceptuses contains a sialylated core type 1 O-linked glycan, while the capsule of the postfixation conceptus contains a desialylated core type 1 glycan. The loss of sialic acid from the sialylated core type 1 in the capsule appears to be directly related to successful fixation of the conceptus at Day 16, and thus critical to the continuation of pregnancy in horses. Thus far, the underlying mechanism of deglycosylation is unknown, and the description of sialidase 2 expression by the equine embryo is the first possible explanation for this phenomenon. *NEU2* mRNA could be detected in increasing levels from Day-8 to Day-14 conceptuses in both microarray and RT-qPCR analysis, and protein expression was localized to trophoblast cells using IHC. It seems, therefore, that the conceptus itself regulates the timely change of its capsule composition, an event leading to its fixation in the uterus.

Lipocalin and Osteopontin

Interestingly, lipocalin and osteopontin showed a decrease in expression in the current study. Both genes are primarily known for their progesterone-dependent expression by endometrial tissue during pregnancy. Lipocalin, *P19*, mRNA decreased as conceptus development proceeded, which could be confirmed using RT-qPCR. Lipocalin is primarily known as a progesterone-dependent protein secreted by the endometrium into the uterine lumen of the mare. It associates with the embryonic capsule and is transferred through the capsule into the yolk sac fluid via trophoblast cells [34]. Northern blot analysis, a less sensitive technique than RT-qPCR, failed to detect expression of P19 in the conceptus [35]. In the current study, lipocalin mRNA was present in moderate amounts in Day-8 conceptuses and decreased in Day-10, -12, and -14 embryos. The observed decrease in *P19* expression may be a consequence of the high amounts of P19 of uterine origin supplied to the conceptus.

SPP1, known as osteopontin, is an extracellular matrix protein that binds integrins to promote cell-cell attachment and communication; it is also reported to act as an immunomodulator [36]. Osteopontin of uterine origin appears to play a key role in conceptus implantation and maintenance of pregnancy in sheep [37] and pigs [38], while such a clear role has not been defined in equine. *SPP1* mRNA levels showed a pronounced decrease from Day-8 to Day-14 conceptuses on both the microarray and RT-qPCR data. To our knowledge, a developmental stage-dependent decrease in *SPP1* expression by conceptuses has not been reported; given the delayed implantation of the equine conceptus, which does not occur until Day 40 [39], we hypothesize that the reduced expression of osteopontin contributes to the prolonged preimplantation phase.

Solute Carriers and Nutrient Uptake

The equine conceptus is dependent upon nutrient uptake from uterine secretions during the preimplantation period. Secreted proteins make up a substantial portion of the gene products up-regulated in pregnant mares, and contribute to formation of the so-called histiotrophs [13]. A total of 42

members of the solute carrier (SLC) group of membrane transport proteins were differentially regulated between the different stages of conceptus development. A total of 30 transporters showed an increase, and 12 transporters showed a decrease in expression level relative to Day-8 conceptuses. SLC transporters are integral membrane proteins that transport a diverse array of solutes [40]. We hypothesize that these transporters contribute to nutrient exchange between the uterus secreting histiotrophs and the developing conceptus. Given that some transporters were up-regulated and some down-regulated, it seems that each developmental stage expresses a unique subset of transporters to facilitate transfer of nutrients required for conceptus growth.

Apolipoproteins are proteins that bind to lipids in order to form lipoproteins for transport. *APOB*, *APOC2*, *APOE*, *APOM*, and *APOH* are five apolipoproteins identified as up-regulated in the current study. In the developing mouse embryo, it has been suggested that APOB plays an essential role in the transport of lipid nutrients to the developing mouse embryo via the yolk sac-mediated synthesis and secretion of lipoproteins [41]. We suggest that the aopolipoproteins expressed by the equine conceptus exert a similar function, and that the array of apolipoproteins expressed ensures adequate nourishment of the developing embryo.

Counteraction of Trophoblast Invasion

We have previously described the down-regulation of plasminogen in equine endometrium during early pregnancy, and suggested this to be part of a mechanism that counteracts trophoblast invasion [13]. The plasminogen system is one of the proteinases implicated in trophoblast invasion [42]. Here we report the down-regulation of plasminogen activator and antiplasmin precursor, as well as the up-regulation of PAI. Protein expression of PAI1 was confirmed by IHC. PAI1 reactive protein was found to be localized to trophoblast cell. These findings further corroborate the previously made suggestion that the plasminogen system is down-regulated to prevent erosion of the endometrium in the equine pregnancy, which is characterized as noninvasive.

Synthesis and Metabolism of Steroid Hormones

As can be expected, a number of enzymes involved in the synthesis and metabolism of steroid hormones were identified as differentially regulated. The steroidogenic acute regulatory protein (STAR) is a transport protein that regulates cholesterol transfer within the mitochondria, which is the rate-limiting step in the production of steroid hormones. *STAR* showed higher expression in Day-12 and -14 conceptuses compared with Day-8 conceptuses. Human embryonic stem cells express STAR at the mRNA and protein level, and hCG treatment rapidly up-regulates STAR-mediated cholesterol transport [43]. In porcine embryos, *STAR* mRNA is maximal during conceptus elongation, and has been suggested as a major regulator of steroidogenesis [44]. The cytochrome P450 cholesterol side-chain cleavage enzyme (P450SCC) is a mitochondrial enzyme associated with the conversion of cholesterol to pregnenolone, which showed a similar expression pattern to *STAR*. *HSD3B1*, an enzyme playing a crucial role in the biosynthesis of all classes of hormonal steroids, was likewise up-regulated in Day-12 and -14 conceptuses. As previously described by Walters et al. [45], expression of *CYP19A1*, commonly known as aromatase, was detected, with mRNA levels starting to increase in Day-12 equine conceptuses. CYP17A1 is a cytochrome P450 enzyme that acts upon pregnenolone and progesterone, showing an increase in expression in the current study starting in Day-12 conceptuses. Four members of the 17β-hydroxysteroid dehydrogenase family showed lower mRNA levels as conceptus development proceeded, while one member, *HSD17B8*, showed an increase in transcript level in Day-14 conceptuses. Changing 17β-hydroxysteroid dehydrogenase activity has been shown in preimplantation mouse and rat embryos, and has been suggested to regulate the estrone-to-estradiol ratio in the embryo [46]. The equine embryo proper has been shown to convert estrone to estradiol and vice versa, an event that could be regulated by stage-specific expression of 17β-hydroxysteroid dehydrogenases.

Phospholipases

Phospholipases (PLAs) catalyze the hydrolysis of phospholipids; depending on the type of reaction catalyzed, PLAs are categorized into four major classes: A, B, C, and D. The expression of PLA2 in equine conceptuses has been described previously [47]. PLA2 catalyzes the release of arachidonic acid needed for prostaglandin synthesis from membrane lipids. Given the large quantities of prostaglandins produced by the equine conceptus, the expression of PLA2 most likely provides substrate for the production of the latter. PLA2G1B (PLA2α) has been shown to be crucial for ''on-time'' embryo implantation in the mouse [48]. For the first time, the expression of phospholipases of each of the four major groups is described in equine conceptuses. The orchestrated expression of these enzymes seems to be crucial for embryonic development, not only in humans and mice, but also in horses.

To conclude, these results give insight into the changes occurring at the mRNA level during early embryonic development in conceptuses retrieved 8, 10, 12, and 14 days after ovulation. GO classification of transcripts differentially expressed underline the ongoing reciprocal embryo-maternal dialogue, with the majority of up-regulated genes being consistent with cell surface changes (the embryo signaling to the uterine environment) and the response to extracellular stimuli (the embryo responding to uterine stimuli). The data support a previously proposed hypothesis regarding the counteraction of trophoblast invasion [13]. Starting points for more detailed investigations are provided with the description of fibrinogen subunit expression at the mRNA and protein level, and the new concept that the conceptus itself regulates the sialic acid content of its capsule through the expression of sialidase.

REFERENCES

1. Short RV. Implantation and the maternal recognition of pregnancy. In: Wolstenholme GEW, and O'Connor M (eds.), Ciba Foundation Symposium on Foetal Autonomy. London: J&A Churchill; 1969:2–26.
2. Bazer FW, Spencer TE, Ott TL. Interferon tau: a novel pregnancy recognition signal. Am J Reprod Immunol 1997; 37:412–420.
3. Geisert RD, Zavy MT, Moffatt RJ, Blair RM, Yellin T. Embryonic steroids and the establishment of pregnancy in pigs. J Reprod Fertil Suppl 1990; 40:293–305.
4. Ginther OJ. Mobility of the early equine conceptus. Theriogenology 1983; 19:603–611.
5. McDowell KJ, Sharp DC, Grubaugh W, Thatcher WW, Wilcox CJ. Restricted conceptus mobility results in failure of pregnancy maintenance in mares. Biol Reprod 1988; 39:340–348.
6. Stout TA, Allen WR. Role of prostaglandins in intrauterine migration of the equine conceptus. Reproduction 2001; 121:771–775.
7. Watson ED, Sertich PL. Prostaglandin production by horse embryos and the effect of co-culture of embryos with endometrium from pregnant mares. J Reprod Fertil 1989; 87:331–336.
8. Zavy MT, Vernon MW, Sharp DC III, Bazer FW. Endocrine aspects of early pregnancy in pony mares: a comparison of uterine luminal and

peripheral plasma levels of steroids during the estrous cycle and early pregnancy. Endocrinology 1984; 115:214–219.
9. Vanderwall DK, Woods GL, Weber JA, Lichtenwalner AB. Corpus luteal function in nonpregnant mares following intrauterine administration of prostaglandin E(2) or estradiol-17beta. Theriogenology 1994; 42:1069–1083.
10. Woodley SL, Burns PJ, Douglas RH, Oxender WD. Prolonged interovulatory interval after oestradiol treatment in mares. J Reprod Fertil Suppl 1979; (27):205–209.
11. Jeong HJ, Kim HJ, Lee SH, Kwack K, Ahn SY, Choi YJ, Kim HG, Lee KW, Lee CN, Cha KY. Gene expression profiling of the pre-implantation mouse embryo by microarray analysis: comparison of the two-cell stage and two-cell block. Theriogenology 2006; 66:785–796.
12. Klein C, Bauersachs S, Ulbrich SE, Einspanier R, Meyer HH, Schmidt SE, Reichenbach HD, Vermehren M, Sinowatz F, Blum H, Wolf E. Monozygotic twin model reveals novel embryo-induced transcriptome changes of bovine endometrium in the preattachment period. Biol Reprod 2006; 74:253–264.
13. Klein C, Scoggin KE, Ealy AD, Troedsson MH. Transcriptional profiling of equine endometrium during the time of maternal recognition of pregnancy. Biol Reprod 2010; 83:102–113.
14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−delta delta C(T)) method. Methods 2001; 25:402–408.
15. Morey JS, Ryan JC, Van Dolah FM. Microarray validation: factors influencing correlation between oligonucleotide microarrays and real-time PCR. Biol Proced Online 2006; 8:175–193.
16. Amleh A, Nair SJ, Sun J, Sutherland A, Hasty P, Li R. Mouse cofactor of BRCA1 (Cobra1) is required for early embryogenesis. PLoS One 2009; 4: e5034.
17. Matsumoto H, Ezoe K, Fukui E, Yoshizawa M. Breast cancer 1 (Brca1) is a potential marker of implantation-competent blastocysts in mouse. In: Abstracts of the 43rd Annual Meeting of the Society for the Study of Reproduction, July 30–August 3, 2010, Milwaukee, Wisconsin. Biol Reprod 2010; Special Issue: Abstract: 251.
18. Cooke FN, Pennington KA, Yang Q, Ealy AD. Several fibroblast growth factors are expressed during pre-attachment bovine conceptus development and regulate interferon-tau expression from trophectoderm. Reproduction 2009; 137:259–269.
19. Mosesson MW, Siebenlist KR, Meh DA. The structure and biological features of fibrinogen and fibrin. Ann N Y Acad Sci 2001; 936:11–30.
20. Iwaki T, Castellino FJ. Maternal fibrinogen is necessary for embryonic development. Curr Drug Targets 2005; 6;535–539.
21. Iwaki T, Sandoval-Cooper MJ, Paiva M, Kobayashi T, Ploplis VA, Castellino FJ. Fibrinogen stabilizes placental-maternal attachment during embryonic development in the mouse. Am J Pathol 2002; 160:1021–1034.
22. Rubel C, Fernandez GC, Dran G, Bompadre MB, Isturiz MA, Palermo MS. Fibrinogen promotes neutrophil activation and delays apoptosis. J Immunol 2001; 166:2002–2010.
23. Smiley ST, King JA, Hancock WW. Fibrinogen stimulates macrophage chemokine secretion through Toll-like receptor 4. J Immunol 2001; 167: 2887–2894.
24. Ababneh MM, Troedsson MH, Michelson JR, Seguin BE. Partial characterization of an equine conceptus prostaglandin inhibitory factor. J Reprod Fertil Suppl. 2000; (56):607–613.
25. Albihn A, Waelchli RO, Samper J, Oriol JG, Croy BA, Betteridge KJ. Production of capsular material by equine trophoblast transplanted into immunodeficient mice. Reproduction 2003; 125:855–863.
26. Oriol JG, Betteridge KJ, Clarke AJ, Sharom FJ. Mucin-like glycoproteins in the equine embryonic capsule. Mol Reprod Dev 1993; 34:255–265.
27. Maccioni HJ, Daniotti JL, Martina JA. Organization of ganglioside synthesis in the Golgi apparatus. Biochim Biophys Acta 1999; 1437:101–118.
28. Harduin-Lepers A, Vallejo-Ruiz V, Krzewinski-Recchi MA, Samyn-Petit B, Julien S, Delannoy P. The human sialyltransferase family. Biochimie 2001; 83:727–737.
29. Zhao W, Chen TL, Vertel BM, Colley KJ. The CMP-sialic acid transporter is localized in the medial-trans Golgi and possesses two specific endoplasmic reticulum export motifs in its carboxyl-terminal cytoplasmic tail. J Biol Chem 2006; 281:31106–31118.
30. Oriol JG, Sharom FJ, Betteridge KJ. Developmentally regulated changes in the glycoproteins of the equine embryonic capsule. J Reprod Fertil 1993; 99:653–664.
31. Monti E, Preti A, Rossi E, Ballabio A, Borsani G. Cloning and characterization of NEU2, a human gene homologous to rodent soluble sialidases. Genomics 1999; 57:137–143.
32. Koda T, Kijimoto-Ochiai S, Uemura S, Inokuchi J. Specific expression of Neu2 type B in mouse thymus and the existence of a membrane-bound form in COS cells. Biochem Biophys Res Commun 2009; 387:729–735.
33. Arar S, Chan KH, Quinn BA, Waelchli RO, Hayes MA, Betteridge KJ, Monteiro MA. Desialylation of core type 1 O-glycan in the equine embryonic capsule coincides with immobilization of the conceptus in the uterus. Carbohydr Res 2007; 342:1110–1115.
34. Crossett B, Suire S, Herrler A, Allen WR, Stewart F. Transfer of a uterine lipocalin from the endometrium of the mare to the developing equine conceptus. Biol Reprod 1998; 59:483–490.
35. Crossett B, Allen WR, Stewart F. A 19 kDa protein secreted by the endometrium of the mare is a novel member of the lipocalin family. Biochem J 1996; 320(pt 1):137–143.
36. Wang KX, Denhardt DT. Osteopontin: role in immune regulation and stress responses. Cytokine Growth Factor Rev 2008; 19:333–345.
37. Johnson GA, Bazer FW, Jaeger LA, Ka H, Garlow JE, Pfarrer C, Spencer TE, Burghardt RC. Muc-1, integrin, and osteopontin expression during the implantation cascade in sheep. Biol Reprod 2001; 65:820–828.
38. Garlow JE, Ka H, Johnson GA, Burghardt RC, Jaeger LA, Bazer FW. Analysis of osteopontin at the maternal-placental interface in pigs. Biol Reprod 2002; 66:718–725.
39. Allen WR, Stewart F. Equine placentation. Reprod Fertil Dev 2001; 13: 623–634.
40. Hediger MA, Romero MF, Peng JB, Rolfs A, Takanaga H, Bruford EA. The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteins. Pflugers Arch 2004; 447:465–468.
41. Farese RV Jr, Cases S, Ruland SL, Kayden HJ, Wong JS, Young SG, Hamilton RL. A novel function for apolipoprotein B: lipoprotein synthesis in the yolk sac is critical for maternal-fetal lipid transport in mice. J Lipid Res 1996; 37:347–360.
42. Zhang X, Shu MA, Harvey MB, Schultz GA. Regulation of urokinase plasminogen activator production in implanting mouse embryo: effect of embryo interaction with extracellular matrix. Biol Reprod 1996; 54:1052–1058.
43. Stocco DM. StAR protein and the regulation of steroid hormone biosynthesis. Annu Rev Physiol 2001; 63:193–213.
44. Blomberg LA, Zuelke KA. Expression analysis of the steroidogenic acute regulatory protein (STAR) gene in developing porcine conceptuses. Mol Reprod Dev 2005; 72:419–429.
45. Walters KW, Corbin CJ, Anderson GB, Roser JF, Conley AJ. Tissue-specific localization of cytochrome P450 aromatase in the equine embryo by in situ hybridization and immunocytochemistry. Biol Reprod 2000; 62: 1141–1145.
46. Wu JT, Matsumoto PS. Changing 17 beta-hydroxysteroid dehydrogenase activity in preimplantation rat and mouse embryos. Biol Reprod 1985; 32: 561–566.
47. Simpson KS, Adams MH, Behrendt-Adam CY, Baker CB, McDowell KJ. Identification and initial characterization of calcyclin and phospholipase A2 in equine conceptuses. Mol Reprod Dev 1999; 53:179–187.
48. Song H, Lim H, Paria BC, Matsumoto H, Swift LL, Morrow J, Bonventre JV, Dey SK. Cytosolic phospholipase A2alpha is crucial [correction of A2alpha deficiency is crucial] for 'on-time' embryo implantation that directs subsequent development. Development 2002; 129:2879–2889.