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Microarray Analysis of Equine Endometrium at Days 8 and 12 of Pregnancy[1]

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ABSTRACT

Establishment and maintenance of pregnancy in equids is only partially understood. To provide new insights into early events of this process, we performed a systematic analysis of transcriptome changes in the endometrium at Days 8 and 12 of pregnancy. Endometrial biopsy samples from pregnant and nonpregnant stages were taken from the same mares. Composition of the collected biopsy samples was analyzed using quantitative stereological techniques to determine proportions of surface and glandular epithelium and blood vessels. Microarray analysis did not reveal detectable changes in gene expression at Day 8, whereas at Day 12 of pregnancy 374 differentially expressed genes were identified, 332 with higher and 42 with lower transcript levels in pregnant endometrium. Expression of selected genes was validated by quantitative real-time RT-PCR. Gene set enrichment analysis, functional annotation clustering, and cocitation analysis were performed to characterize the genes differentially expressed in Day 12 pregnant endometrium. Many known estrogen-induced genes and genes involved in regulation of estrogen signaling were found, but also genes known to be regulated by progesterone and prostaglandin E2. Additionally, differential expression of a number of genes related to angiogenesis and vascular remodeling suggests an important role of this process. Furthermore, genes that probably have conserved functions across species, such as *CRYAB*, *ERRFI1*, *FGF9*, *IGFBP2*, *NR2F2*, *STC1*, and *TNFSF10*, were identified. This study revealed the potential target genes and pathways of conceptus-derived estrogens, progesterone, and prostaglandin E2 in the equine endometrium probably involved in the early events of establishment and maintenance of pregnancy in the mare.

embryo-maternal communication, equus caballus, *female reproductive tract, gene regulation, horse, pregnancy, steroid hormones, uterus*

INTRODUCTION

Progesterone produced from a viable corpus luteum is essential for establishment and maintenance of pregnancy. In the mare, cyclical luteolysis takes place between Days 14 and 16 after ovulation. The equine conceptus must therefore prevent luteal regression, a process commonly referred to as maternal recognition of pregnancy. In contrast to other large domestic animal species, the nature of embryo-maternal communication and maternal recognition of pregnancy in equids is still not completely understood. Furthermore, a number of features of equine pregnancy are unique to the genus *Equus* and differ from corresponding events in other mammals.

The equine blastocyst enters the uterus between 144 and 156 h after ovulation [1]. Between Day 7 and Day 21, the embryo is completely enveloped by a tough glycoprotein capsule, which prevents the trophoblast from elongating and provides its typical spherical shape [2, 3]. Furthermore, the capsule is thought to play a protective role, to ensure nutrition, and to facilitate migration of the equine conceptus [4]. The capsule may also concentrate growth factors at the embryo-maternal interface and eventually release them in a controlled manner [5]. Until Day 16, the equine conceptus remains completely unattached within the uterus and migrates continuously throughout the uterine lumen driven by peristaltic myometrial contractions [6, 7]. The constant movement allows the embryo to get in contact with most of the endometrial surface, likely serving to signal its presence uniformly to the entire endometrium and to garner uterine secretions [8]. At Day 17, not only as a result of increased conceptus diameter and increased uterine tone, but also because of changes in the embryo's capsule and uterine environment, the conceptus becomes immobilized (''fixed'') at the base of one of the uterine horns [6, 9, 10].

Although the mechanisms of luteal rescue in the mare are still unknown, the role of prostaglandins is undisputed. In cyclic mares luteolysis is triggered by an oxytocin-dependent pulsatile release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the endometrium from Day 14 after ovulation [11]. However, in the presence of a conceptus, the synthesis and secretion of $PGF_{2\alpha}$ in the mare is abrogated [8]. Furthermore, coincubation of conceptus membranes with endometrial tissue has been shown to block $PGF_{2\alpha}$ production in vitro [12]. Although the signal that accomplishes this effect is not known, the presence of a conceptus seems to uncouple the oxytocin-induced release of $PGF_{2\alpha}$ [8, 13]. It has been demonstrated that the $PGF_{2\alpha}$ response to oxytocin is maximal at the time of luteolysis in nonpregnant mares and that this response cannot be induced during early pregnancy either with endogenous or with exogenous oxytocin [13–15]. These data suggest that maternal recognition of pregnancy, which in the mare is commonly

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believed to occur between Days 14 and 16 [16], may be as early as Days 11–13 [13].

Another hypothesis is that the antiluteolytic signal produced by the equine conceptus targets prostaglandin biosynthesis in order to prevent luteolysis. Prostaglandin G/H synthase 2 (PTGS2; also known as cyclooxygenase 2), a rate-limiting enzyme in prostaglandin synthesis, has been shown to be up-regulated at Day 14/15 of the estrous cycle, but not at corresponding days in pregnant mares [17, 18]. Moreover, *PTGS2* mRNA abundance and $PGF_{2\alpha}$ concentrations have been shown to be reduced by conceptus secretions in an equine endometrial explant culture system [18]. Therefore it has been suggested that the conceptus blocks endometrial $PGF_{2\alpha}$ synthesis at least in part by repressing the induction of *PTGS2* expression.

What also remains unknown is the nature of the embryonic pregnancy recognition signal to prevent luteolysis. The equine conceptus produces a number of different secretory products during early pregnancy, including steroids, prostaglandins, different proteins, and peptides [19], such as interferon delta, a member of the type I interferon family [20]. Moreover, the application of intrauterine devices has been demonstrated to prolong the luteal phase in the mare, indicating that a form of mechanotransduction by the migrating conceptus may prevent the endometrial cells from releasing $PGF_{2\alpha}$ [21].

In order to systematically analyze the maternal response, i.e., the changes in the equine endometrium, to the presence of a conceptus a transcriptome study of endometrium samples from six mares at Days 8 and 12 of pregnancy and the corresponding nonpregnant stages was performed.

MATERIALS AND METHODS

Sample Collection and Experimental Design

In this study, two experiments were performed. Endometrial biopsy samples were collected from inseminated mares 1) on Day 8 and 2) on Day 12 after ovulation. In both experiments one pregnant and one control (nonpregnant) sample were taken from every mare by random order. Only one endometrial biopsy was taken per estrous cycle.

Samples were collected from six normal cycling Bavarian Warmblood mares belonging to the Bavarian principal and state stud of Schwaiganger, Germany. Follicular development and ovulation were monitored routinely by daily transrectal palpation and ultrasound examination. When mares developed an ovarian follicle of approximately 35 mm in diameter, accompanied by prominent endometrial edema, they were treated with 1500 IU human chorionic gonadotropin i.v. (Ovogest; Intervet Deutschland GmbH, Unterschleissheim, Germany) to induce ovulation. All mares were inseminated artificially with $>500 \times 10^6$ freshly collected, progressively motile, extended spermatozoa from one fertile stallion. Insemination was performed 24 h after induction of ovulation and was repeated if ovulation had not occurred after 48 h. Endometrial samples were obtained by transcervical biopsy. Samples were collected 1) on Day 8 and 2) on Day 12 after flushing of the uterus. On Day 8, mares were rated pregnant if embryo recovery was successful. On Day 12, pregnancy was additionally proved by ultrasonographic detection of an embryonic vesicle in the uterine lumen before flushing. Embryos were flushed transcervically without sedation using up to four times 1.5 L prewarmed and sterile filtered phosphate buffered saline (Lonza Verviers Sprl, Verviers, Belgium). The fluid was recovered directly into sterile glass bottles and subsequently, if necessary, filtered with an embryo filter system and examined under a microscope (in the case of Day 8 embryos) for the presence of an embryo.

For determination of peripheral plasma progesterone (P4) concentrations, blood samples were collected in ethylenediaminetetraacetic acid tubes from the jugular vein on Day 0 and directly after biopsy. Blood samples were centrifuged at $2000 \times g$ for 10 min and plasma was decanted and stored at −20°C until assay.

In order to analyze tissue composition, the biopsy samples were cut transversely into six equal and plane-parallel slices. For quantitative stereological analyses, every second slice was transferred into embedding capsules with their right cut surface facing downwards, covered with a foam sponge to avoid distortion of the tissue samples, and fixed by immersion in 4% buffered formaldehyde. The remaining pieces of the biopsy samples were immediately transferred into vials containing 4 ml RNAlater (Ambion, Huntingdon, U.K.) for mRNA expression analysis. The vials were cooled on ice and incubated overnight at 4°C. Samples were stored at −80°C until further processing. All experiments with animals were conducted with permission from the local veterinary authorities and in accordance with accepted standards of humane animal care.

Quantitative Stereological Analysis

For qualitative histological and quantitative stereological analyses, three formalin-fixed slices of each biopsy sample were routinely processed and embedded in paraffin with their right cut surface facing downwards. Histological sections were cut at a nominal thickness of 3 μm with a rotary microtome, transferred onto glass slides, and stained with hematoxylin and eosin (H&E). Quantitative stereological analyses were carried out with newCAST software (Visiopharm A/S, Hoersholm, Denmark). Slides were displayed on a monitor at 400× final magnification via a camera (universal camera DP72, Olympus Deutschland GmbH, Hamburg, Germany) coupled to a microscope (standard laboratory microscope BX41, Olympus Deutschland GmbH) and images were superimposed by an adjustable point counting grid. More than 7000 points were evaluated per biopsy sample to determine the volume densities of surface epithelium, glandular epithelium, blood vessels, and remaining tissue. The volume densities (Vv) of the different tissue compartments were obtained by dividing the number of points hitting a compartment ($P_{(compartment)}$, e.g., points hitting blood vessels, $P_{(blood\ vessels)}$) by the total number of points hitting the biopsy sample ($P_{(sample)}$): $Vv_{(compartment/sample)} = P_{(compartment)}/P_{(sample)}$.

Microarray Analysis

Total RNA was isolated from the 12 endometrial biopsy samples using Trizol reagent (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions. Quantity and purity of RNA were measured with a NanoDrop 1000 (PEQLAB Biotechnologie GMBH, Erlangen, Germany). Quality of total RNA was determined electrophoretically with an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). RNA integrity values ranged from 8.3 to 9.2. Microarray analysis was performed using Agilent 4x44k Horse Gene Expression microarrays (AMADID 021322). Cy3-labeled cRNA was produced with the Quick Amp Labeling Kit, one-color (Agilent Technologies), and hybridized to the microarrays according the manufacturer's instructions. Hybridized and washed slides were scanned at 3-μm resolution with an Agilent DNA Microarray Scanner (G2505C; Agilent Technologies). Image processing was performed with Feature Extraction Software 10.5.1.1 (Agilent Technologies). Processed signals were filtered based on ''Well above background'' flags (detection in four of six samples in either one of the two experimental groups) and subsequently normalized with the BioConductor package vsn [22]. For quality control normalized data was analyzed with a distance matrix and a heatmap based on pair-wise distances (BioConductor package geneplotter). Significance analysis was performed using the Microsoft Excel add-in ''Significance analysis of microarrays'' (SAM, two-class paired) [23]. Significance thresholds were set as follows: 1) false discovery rate (FDR) <5% and fold change at least 1.5-fold and 2) ratio fold change/q-value ≥0.75 to have higher confidence for smaller differences. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE21046.

Functional Analysis of Array Data

The Agilent horse microarray was reannotated based on Ensembl 55, Entrez Gene, and BLAST analyses to obtain equine and human (putative orthologous genes) Entrez Gene identifiers and the corresponding gene information. For gene set enrichment analysis (GSEA) [24], genes were preranked based on fold change pregnant vs. control and SAM q-value (log2(fold change + 2) * −log10(q-value)). This preranked gene list was compared with GSEA gene sets c2.all.v2.5.symbols.gmt (curated) and our own published and unpublished gene sets (see *Results*). Functional classification of differentially expressed genes (DEGs) was done with the ''Functional annotation clustering'' and ''Functional annotation chart'' tools of the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [25] and the text-mining tool CoPub [26], which finds biomedical concepts from Medline that are significantly linked to the gene set. Both analyses were performed on the basis of Entrez Gene IDs of the putative human orthologous genes. Interaction networks were drawn with the Pathway Architect software (version 3.0.1; Stratagene, Heidelberg, Germany).

Quantitative Real-Time RT-PCR

The same RNA samples as for microarray analysis were used for quantitative real-time RT-PCR (qPCR). First-strand cDNA was synthesized

starting from 1 μg total RNA with the Sprint RT Complete-Double PrePrimed Kit (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). The two-step quantitative real-time PCR experiments were performed as described previously [27] in accordance with the MIQE guidelines [28]. The LightCycler DNA Master SYBR Green I protocol (Roche, Mannheim, Germany) was applied. Primer sequences, annealing temperatures (AT), the appropriate fluorescence acquisition (FA) points for quantification within the fourth step of the amplification segment, and the melting points (MP) are shown in Supplemental Table S1 (all Supplemental Data are available online at www.biolreprod.org). The cycle number (CT) required to achieve a definite SYBR Green fluorescence signal was calculated by the second derivative maximum method (LightCycler software version 3.5.28). The CT is correlated inversely with the logarithm of the initial template concentration. The CT determined for the target genes were normalized against the geometric mean of the housekeeping genes histone (H3F3A), ubiquitin (UBQ3), and 18S rRNA (ΔCT) [29]. Finally, with respect to the paired design, the relative expression difference between the nonpregnant and pregnant state was calculated for each animal (ΔΔCT). All amplified PCR fragments were sequenced to verify the resulting PCR product.

Progesterone Assay

Progesterone concentrations in peripheral blood plasma were measured with a mini VIDAS (bioMérieux Deutschland GmbH, Nürtingen, Germany) and VIDAS Progesterone kits, a system based on the enzyme-linked fluorescent assay technique. A detection limit of 0.25 ng/ml and a correlation coefficient of 0.89 towards radio immune assay are certified for the assay by the manufacturer.

RESULTS

To characterize endometrial responses to the early embryo in the mare, microarray analyses of Day 8 and Day 12 endometrial biopsy samples were performed in two separate experiments. A paired design was used, i.e., RNA samples derived from the same mare were hybridized on the same slide (4x44k array) to reduce technical and biological variation. The paired design was chosen to take into account potential interindividual differences related to genetic background and other actors. Additional sources for variation were tried to rule out with the measurement of P4 concentrations and the analysis of the composition of the endometrial biopsy samples.

Peripheral Plasma Progesterone Concentrations

P4 values showed basal levels on Day 0. On Day 8, plasma progesterone concentrations ranged from 12.6 to 27.7 ng/ml and on Day 12 from 12.0 to 35.3 ng/ml. Plasma progesterone concentrations were not significantly different between pregnant and nonpregnant mares on Day 8 and on Day 12, respectively (*t*-test: $P > 0.05$; data not shown).

Quantitative Stereological Analysis

Tissue composition of all endometrial biopsy samples, i.e., the volume fractions of luminal epithelium (LE), blood vessels (BV), glandular epithelium (GE), and remaining tissue (Rest), was determined by using quantitative stereological techniques (Supplemental Figs. S1 and S2). Overall, tissue composition was quite consistent within the biopsy samples (see examples in Supplemental Fig. S2).

In endometrial biopsy samples collected on Day 8, volume fractions of the different structures were 0.23%–0.91% (LE), 2.4%–3.9% (BV), 25.8%–35.8% (GE), and 59.3%–71.3% (Rest). Maximal deviation was 0.41 percentage points (pp) (LE), 1.3 pp (BV), 4.8 pp (GE), and 3.6 pp (Rest) within pregnant and control samples of one mare.

In endometrial biopsy samples collected on Day 12, volume fractions of the different structures were 0.24%–1.82% (LE), 2.7%–3.9% (BV), 22.9%–33.3% (GE), and 62.4%–73.7% (Rest). Maximal deviation was 0.54 pp (LE; excluding mare #3), 0.8 pp (BV), 7.7 pp (GE), and 8.6 pp (Rest) within pregnant and control samples of one mare. In mare #3, volume fraction of LE was 1.5 pp higher (5.6-fold) in the control sample than in the pregnant sample.

Microarray Analysis

After data processing and normalization the microarray data sets were initially analyzed with correlation heatmaps in order to cluster the data sets of the individual samples according to their pair-wise correlations. Then statistical analysis was done to identify DEGs. For the endometrial tissue samples derived from Day 8 pregnant mares vs. Day 8 control mares, statistical analysis did not reveal any significant expression differences (data not shown), even after exclusion of mare #3 (aberrant expression differences for immune response genes in pregnant sample).

In contrast to Day 8, differential gene expression was identified at Day 12 of pregnancy. A heatmap of pair-wise correlations based on normalized microarray data sets is shown in Figure 1a for analysis of Day 12 of pregnancy. Samples from the same mares clustered together, but no grouping could be observed within samples collected during pregnancy or during the estrous cycle. The control sample of mare #3 (Fig. 1a, M3 co) showed the lowest correlation to all other samples. A second heatmap was generated based on a limited number of hybridization probes, which showed at least 1.5-fold difference between pregnant and control samples (Fig. 1b). Based on this reduced data set a clear separation of pregnant and control samples was obtained. Figure 1c shows a heatmap of log2 fold changes pregnant vs. control for the six mares. Except for mare #3, similar expression patterns were observed between mares. For mare #3, many genes showed inverse expression differences. Because of the 5.6-fold higher proportion of luminal epithelium in the control sample compared to the pregnant sample (Supplemental Fig. S2) and the results of the heatmap analysis (Fig. 1c), data from mare #3 were excluded from further analysis. Statistical analysis of Day 12 microarray data of the remaining five mares revealed 374 DEGs in endometrial tissue samples of pregnant vs. control mares (Supplemental Table S2). Of these genes, 332 transcripts showed at least 1.5-fold higher expression values (in the following referred to as up-regulated genes) and 42 transcripts showed lower expression values (in the following referred to as down-regulated genes) in biopsy samples from pregnant endometrium compared to control samples. Figure 1c shows a cluster analysis of log2 fold changes of the DEGs for all six mares. Whereas similar pregnant to control expression differences were observed for five of the mares, mare #3 showed for many of these genes either no expression differences or even inverse differences (Fig. 1c, M3).

Differential expression was in addition analyzed between Day 8 and Day 12 control samples (see Supplemental Table S2). Of the Day 12 DEGs (pregnant vs. control), 34 genes were also differentially expressed in Day 12 compared to Day 8 control samples (fold change >1.5-fold, FDR 5%): 6 of the Day 12 down-regulated genes and 28 of the up-regulated genes. Most of the Day 12 of pregnancy down-regulated genes (5 of 6) showed lower mRNA levels in Day 12 vs. Day 8 control samples. Likewise there were a number of genes up-regulated from Day 8 to Day 12 in the control samples that were additionally up-regulated in Day 12 pregnant samples. Furthermore, there were some genes down-regulated from Day 8 to Day 12 of the estrous cycle but with higher mRNA levels in Day 12 pregnant compared to Day 12 control samples.

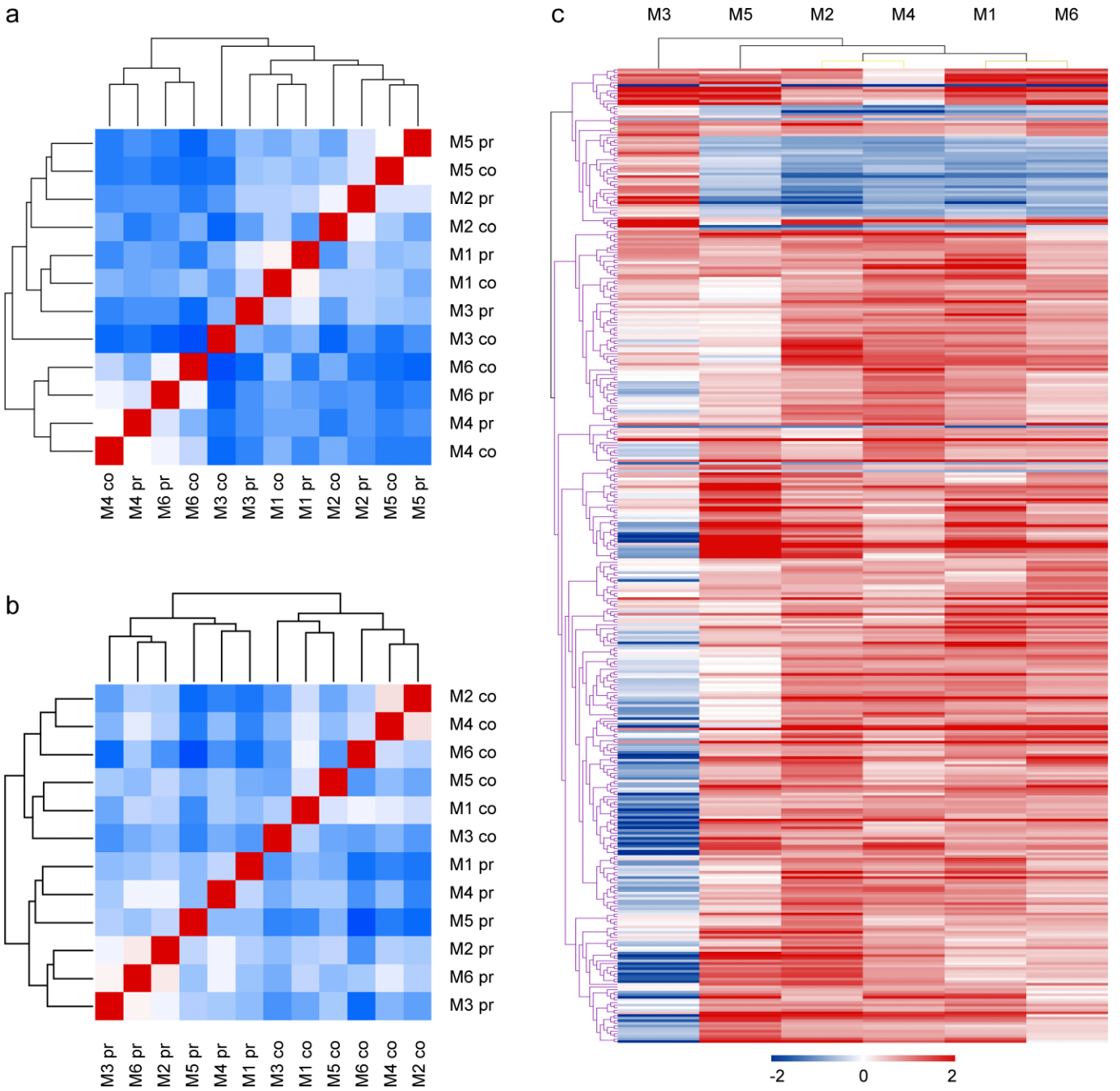

FIG. 1. Microarray analysis of Day 12 pregnant vs. nonpregnant endometrium. Normalized expression data was clustered based on pair-wise correlation using all detectable probes (**a**) and after filtering for probes with at least 1.5-fold mean difference between pregnant and control samples (**b**) (red: correlation = 1; blue: lowest observed correlation). After statistical analysis a hierarchical cluster analysis of the log2 fold changes of the single mares limited to the significant genes was performed (**c**). Mare #3 is also shown but was excluded from the statistical analysis. M, mare #; pr, pregnant; co, control.

Validation of Microarray Results by Quantitative Real-Time RT-PCR

To validate microarray results, 13 of the DEGs were selected for quantification with real-time RT-PCR (Table 1). Overall, expression differences found by microarray analysis were confirmed. For some of the analyzed genes *t*-test *P*-values were not significant (>0.05) because of variations in expression differences between mares. For most of those genes, expression differences were significant between Day 8 and Day 12 pregnant samples (Table 2). The comparison of qPCR data between Days 8 and 12 corresponded well to the array data and showed that four of the analyzed genes (*CTSL1*, *FGF9*, *PTGR1*, *SLC36A2*) were also differentially expressed between Days 8 and 12 of the estrous cycle (Table 2). Interestingly, *FGF9* was down-regulated at Day 12 of the estrous cycle compared to Day 8 of the estrous cycle. Samples derived from mare #3 were also analyzed, and the findings of the microarray experiment that for many of the DEGs expression differences were much lower or even inverse were confirmed (data not shown).

TABLE 1. Quantification of selected genes with quantitative real-time RT-PCR: Day 12 pregnancy vs. control.

Gene name	Gene symbol	Entrez gene ID	Ensembl gene ID	Hsa Gene symbol	Hsa Entrez gene ID	Qpcr		Array	
						Pr/Co[a]	*P*-value	Pr/Co[a]	q-value
Cathepsin L	*LOC100061532*	100061532	ENSECAG00000007210	*CTSL1*	1514	−2.7	0.002	−2.2	0.012
ERBB receptor feedback inhibitor 1	*ERRFI1*	100052062	ENSECAG00000017104	*ERRFI1*	54206	1.7	0.012	2.6	0.009
Fibroblast growth factor 9	*LOC100050353*	100050353	ENSECAG00000018716	*FGF9*	2254	7.9	0.017	8.8	0.001
Hedgehog-interacting protein	*HHIP*	100062868	ENSECAG00000024485	*HHIP*	64399	−1.6	0.090	−1.7	0.001
Kinase insert domain receptor	*KDR*	100033959	ENSECAG00000019429	*KDR*	3791	1.8	0.061	1.7	0.012
Krueppel-like factor 9	*KLF9*	100050300	ENSECAG00000024925	*KLF9*	687	1.3	0.179	1.5	0.018
Oxytocin receptor	*LOC100058848*	100058848	ENSECAG00000017844	*OXTR*	5021	1.8	0.050	1.6	0.018
Progestin and adipoQ receptor family member V	*LOC100064749*	100064749	ENSECAG00000008154	*PAQR5*	54852	4.7	0.001	2.0	0.002
Prostaglandin E2 receptor EP4 subtype	*LOC100053208*	100053208	ENSECAG00000011145	*PTGER4*	5734	2.2	0.001	2.0	<0.001
Prostaglandin reductase 1	*PTGR1*	100058059	ENSECAG00000004698	*PTGR1*	22949	3.0	0.069	2.7	0.018
Secreted frizzled-related sequence protein 1	*LOC100055845*	100055845	ENSECAG00000021358	*SFRP1*	6422	1.5	0.147	1.7	0.012
Solute carrier family 36 (proton/amino acid symporter), member 2	*LOC100071541* 3′-UTR[b]	100071541	ENSECAG00000011961	*SLC36A2*	153201	53.1	<0.001	84.3	<0.001
Solute carrier family 36 (proton/amino acid symporter), member 2	*LOC100071541* ORF[c]	100071541	ENSECAG00000011961	*SLC36A2*	153201	32.2	<0.001	2.5	<0.001
Solute carrier organic anion transporter family member 2A1	*SLCO2A1*	100065438	ENSECAG00000024948	*SLCO2A1*	6578	1.8	0.102	2.0	0.021

[a] Pr: pregnant; Co: control.
[b] UTR, untranslated region.
[c] ORF, open reading frame.

Bioinformatics Analysis of Microarray Data

In order to get a first characterization of the DEGs, the Day 12 expression data set was ranked according to the expression fold change and the SAM q-value (see *Materials and Methods*), resulting in a ranked gene list containing the most significantly up-regulated genes on Day 12 of pregnancy at the top and the most significantly down-regulated genes at the bottom of the list. This preranked list was compared to gene sets of the GSEA Molecular Signature Database, of selected published studies, and of our own published and unpublished studies. Table 3 shows a number of significantly enriched gene sets, i.e., sets with genes occurring toward the top of the preranked Day 12 gene list. The corresponding enrichment plots are shown in Supplemental Figure S3. The gene set with the highest enrichment score and 24 (of 63) overlapping genes in ranks 1–500 of the Day 12 preranked gene list contains genes up-regulated in equine endometrium at Day 13.5 of pregnancy [30]. This gene set is followed by a set of genes up-regulated in human endometrium 7 days after the LH surge (the window of implantation) compared to 2 days after the LH surge [31] (29 of 129 genes in top 500). The gene set with the largest number of overlapping genes within ranks 1–500 was Boquest_CD31$^+$_vs_CD31$^-$_up (75 of 540 genes). Significant enrichment was also found for the corresponding gene set Boquest_CD31$^+$_vs_CD31$^-$_dn (38 of 215 genes). These gene sets were obtained from a comparison of two populations of CD45$^-$CD34$^+$CD105$^+$ adipose tissue-derived adult stromal stem cells that were either CD31 (PECAM1) positive or negative [32]. In addition, gene sets containing hypoxia-induced genes, genes of the RAS pathway, TGF-beta-induced genes, targets of the transcription factor TCF21, vascular endothelial growth factor (VEGF)-induced genes, estrogen-induced genes [33–36], genes up-regulated in ovine endometrium between Days 9 and 12 of pregnancy [37], and prostaglandin E2 (PGE2)-induced genes were found as significantly enriched. The analysis of gene sets from our own studies of bovine and porcine endometrium revealed best enrichment scores for genes up-regulated at Day 14 of pregnancy in porcine endometrium [38] and at Day 18 of pregnancy in bovine endometrium (our unpublished data) but the number of genes in ranks 1–500 of the Day 12 preranked list was rather small (23 and 25 genes, respectively). Higher numbers of genes in ranks 1–500 were found for the gene sets ''up-regulated at estrus in bovine endometrium'' (58 genes) and ''up-regulated at diestrus in bovine endometrium'' (44 genes). Additional information for the gene sets and the genes overlapping with the top 500 of the Day 12 preranked gene list can be found in Supplemental Table S3.

In the next step the up-regulated genes of ranks 1–500 were sorted based on their frequencies: 1) in the gene sets ''Up-regulated in human endometrium during the window of implantation'' (two human gene sets were combined), ''Up-regulated at Day 14 of pregnancy in porcine endometrium,'' and ''Up-regulated at Day 18 of pregnancy in bovine endometrium''; 2) in the gene sets ''Up-regulated in ovine endometrium between Days 9 and 12 of pregnancy,'' and ''Up-regulated at diestrus in bovine endometrium''; and 3) in the gene sets ''Up-regulated at estrus in bovine endometrium'' and ''Estrogen-induced genes'' to find genes that have conserved functions across mammalian species regarding establishment and maintenance of pregnancy. The genes anterior gradient homolog 2 (*AGR2*, Pr/Co = 1.6, q-value = 0.0348, rank 433), G protein-coupled receptor, family C, group 5, member B (*GPRC5B*, Pr/Co = 1.13, q-value = 0.0264, rank 480), ubiquitin D (*UBD*, Pr/Co = 1.4, q-value = 0.025, rank 395), and ubiquitin-conjugating enzyme E2L 6 (*UBE2L6*, Pr/Co = 1.2, q-value = 0.021, rank

TABLE 2. Quantification of selected genes with quantitative real-time RT-PCR: Day 12 vs. Day 8.[a]

	qPCR Co 12/8		Array Co 12/8		qPCR Pr 12/8		Array Pr 12/8	
Gene symbol	FC	*P*-value	FC	q-value	FC	*P*-value	FC	q-value
CTSL1	−2.6	0.025	−2.7	0.010	−8.4	<0.001	−7.0	<0.001
ERRFI1	1.3	0.489	1.5	0.148	2.3	0.002	3.6	<0.001
FGF9	−2.2	0.007	−1.6	0.019	4.4	0.040	5.9	<0.001
HHIP	−1.1	0.589	1.1	0.435	−1.6	0.063	−1.3	0.137
KDR	1.1	0.490	1.1	0.341	2.3	0.011	1.9	0.003
KLF9	1.1	0.596	1.1	0.272	1.5	0.108	1.5	0.229
OXTR	1.1	0.896	1.2	0.339	2.2	0.032	1.9	0.006
PAQR5	2.1	0.370	1.0	0.511	14.3	0.012	2.2	0.007
PTGER4	1.1	0.829	1.1	0.359	2.6	0.003	2.4	<0.001
PTGR1	4.2	0.020	1.7	0.054	12.9	0.001	3.9	<0.001
SFRP1	1.1	0.855	1.2	0.245	1.5	0.126	1.9	0.014
SLC36A2 3′-UTR[b]	2.9	0.029	3.6	0.012	144.2	<0.001	198.0	<0.001
SLC36A2 ORF[c]	2.2	0.024	−1.1	0.312	93.7	<0.001	2.1	0.001
SLCO2A1	1.2	0.567	1.1	0.384	2.4	0.032	2.0	0.026

[a] Pr: pregnant; Co: control; FC: fold change.
[b] UTR, untranslated region.
[c] ORF, open reading frame.

401) matched two gene sets containing up-regulated genes during pregnancy and one genes set up-regulated by progesterone, but these genes showed no significant up-regulation according to the thresholds of the significance analysis. B-cell CLL/lymphoma 6 (*BCL6*, Pr/Co = 1.7, q-value = 0.039, rank 453), crystallin, alpha B (*CRYAB*, Pr/Co = 2.2, q-value = 0.003, rank 85), insulin-like growth factor binding protein 2 (*IGFBP2*, Pr/Co = 1.9, q-value = 0.002, rank 74) and stanniocalcin 1 (*STC1*, Pr/Co = 3.1, q-value = 0.0001, rank 10) matched two gene sets containing up-regulated genes during pregnancy. Insulin-like growth factor binding protein 1 (*IGFBP1*, Pr/Co = 5.8, q-value = 0.004, rank 50) matched one gene set containing up-regulated genes during pregnancy and two gene sets up-regulated by progesterone. A list of all genes and their frequencies in the gene sets is shown in Supplemental Table S4.

To find quantitatively enriched functional terms for the Day 12 up-regulated genes, the DAVID functional annotation clustering tool was used. This method clusters significantly enriched functional terms, i.e., significantly more differential genes were found for a given term than expected, which contain similar sets of genes. This analysis resulted in a relatively large number of significant clusters of related functional terms that represented a variety of biological themes (Supplemental Table S5). These quantitatively enriched biological themes or processes included glycoproteins, secretory proteins, membrane proteins, development, differentiation, angiogenesis, calcium ion binding, carbohydrate binding, wound healing, apoptosis, cell migration, tissue remodeling, neurogenesis, cell growth, and proliferation. The text mining tool CoPub that identifies biological keywords from the Medline database significantly linked to a given gene set from a microarray data analysis [26] also highlighted a list of keywords that were significantly correlated with the genes up-regulated at Day 12 of pregnancy (Supplemental Table S6). The obtained keywords confirmed the results of DAVID functional annotation clustering and included a number of additional terms such as chemotaxis, inflammation, cell adhesion, cell invasion, cytoskeleton, different reproduction-related terms, and endocytosis.

Expression of Genes Involved in Prostaglandin Signaling and Metabolism

Microarray analysis revealed several up-regulated genes in Day 12 pregnant endometrium with a significant fold change ranging from 1.6 to 2.7 that are known to play a role in prostaglandin signaling and metabolism. In particular, transcripts for prostaglandin E receptors 3 and 4 (PTGER3, PTGER4), genes similar to prostaglandin F synthase (LOC100070491, LOC100070501), a prostaglandin transporter, and a prostaglandin reductase were found (Table 4). In addition to these differentially expressed prostaglandin-related genes, many more transcripts of genes involved in prostaglandin signaling and metabolism were found to be expressed in equine endometrium on Day 12 but were not differentially expressed according to the thresholds applied in the statistical analysis (Supplemental Table S7).

Angiogenesis and Steroid Hormone/Prostaglandin Signaling Interaction Networks

Putative interaction networks for genes related to the process of angiogenesis (Fig. 2) and genes described in context of steroid hormone and prostaglandin signaling (Fig. 3), were generated based on a literature search, CoPub results, and interactions from the Pathway Architect database and other public protein interaction databases. For the process of angiogenesis, genes representing different levels of angiogenesis regulation were found, such as members of the angiopoietin family, members of the VEGF system, hypoxia-induced genes, and genes regulating endothelial cell fate (Fig. 2 and Supplement to Fig. 2). The interaction network related to steroid hormone and prostaglandin signaling was clearly dominated by estradiol (E2) with many E2-regulated genes (Fig. 3 and Supplement to Fig. 3). There were also a number of genes described as negative regulators of estrogen receptor 1 (ESR1), genes involved in regulation of growth and differentiation, and genes involved in E2 metabolism. A considerable number of genes were involved in both networks.

Day 12 of Pregnancy Down-Regulated Genes

For the down-regulated genes, quantitatively enriched functional terms were obtained neither with DAVID Functional Annotation Clustering nor with CoPub. The down-regulated genes belonged to very different functional classes. The five most down-regulated genes were FXYD domain-containing ion transport regulator 4 (FXYD4, −3.4), keratin 4 (KRT4, −2.8), cartilage acidic protein 1 (CRTAC1, −2.4), RELT-like 2 (RELL2, −2.2), and cathepsin L1 (CTSL1, −2.2).

TABLE 3. Selected results of Gene Set Enrichment Analysis.

Gene set	Size[a]	NES[b]	FDR q-value[c]	FWER *P*-value[d]	Rank at max	Rank in top 500[e]	Rank in top 250[f]
Genes up-regulated at Day 13.5 of pregnancy in equine endometrium [30]	63	3.17	0.0000	0.0000	634	24	21
Genes up-regulated in human endometrium LH+7 vs. LH+2 [31]	122	2.90	0.0000	0.0000	1283	29	17
Boquest_CD31^{+}_vs_CD31^{-}_up — genes associated with endothelium, related to MHC class II complex and antigen presentation, genes for cytokines and cytokine receptors, and genes involved in signal transduction and transcription	540	2.69	0.0000	0.0000	2046	75	37
Boquest_CD31^{+}_vs_CD31^{-}_dn — genes involved in cell cycle arrest, stem cell biology and development, and in biology of adipose tissue, bone, cartilage, muscle, and neuronal tissue	215	2.64	0.0000	0.0000	2465	38	20
Manalo_hypoxia_up — genes up-regulated in human pulmonary endothelial cells under hypoxic conditions	84	2.60	0.0000	0.0000	2169	16	8
Genes up-regulated at Day 14 of pregnancy in porcine endometrium [38]	131	2.40	0.0000	0.0000	1746	23	14
Genes up-regulated at Day 18 of pregnancy in bovine endometrium[g]	226	2.37	0.0001	0.0010	2815	25	12
RAS_oncogenic_signature — gene expression signature that reflects the activity of the RAS-induced pathway	200	2.30	0.0003	0.0080	2601	25	14
TGFbeta_all_up — up-regulated by TGF-beta treatment of skin fibroblasts	73	2.30	0.0003	0.0090	1447	16	10
Genes up-regulated at estrus in bovine endometrium[h]	462	2.29	0.0004	0.0100	1815	58	34
Estrogen-induced genes[i]	400	2.29	0.0004	0.0100	1575	47	21
Genes up-regulated in receptive (LH+8) vs. pre-receptive (LH+3) human endometrium [41]	44	2.27	0.0000	0.0000	2778	11	6
Pod1_KO_dn — down-regulated in glomeruli isolated from Pod1 (TCF21)$^{-/-}$ mice versus wild-type controls	592	2.24	0.0005	0.0180	2576	53	27
Genes up-regulated at diestrus in bovine endometrium[h]	466	2.19	0.0009	0.0400	2731	44	25
VEGF_MMMEC_all_up — VEGF-induced genes in human myometrial microvascular endothelial cells	84	2.17	0.0011	0.0550	1949	14	7
Genes up-regulated in ovine endometrium between Days 9 and 12 of pregnancy [37]	358	2.07	0.0004	0.0010	2947	27	12
PGE2 up-regulated genes in human monocyte-derived dendritic cells [j]	121	1.92	0.0004	0.0060	2439	16	9

[a] Number of genes in a gene set that matched with the ranked gene list.
[b] NES, normalized enrichment score.
[c] FDR, false discovery rate.
[d] FWER, family-wise error rate.
[e] Genes in top 500 of pre-ranked gene list.
[f] Genes in top 250 of pre-ranked gene list.
[g] Affymetrix analysis of bovine endometrium from Day 18 pregnant animals vs. Day 18 controls.
[h] Affymetrix analysis of bovine endometrium estrus vs. diestrus.
[i] Estrogen-induced genes derived from different data sets (see *Results* [33–36]).
[j] Gene set derived from GEO gene expression series GSE8539.

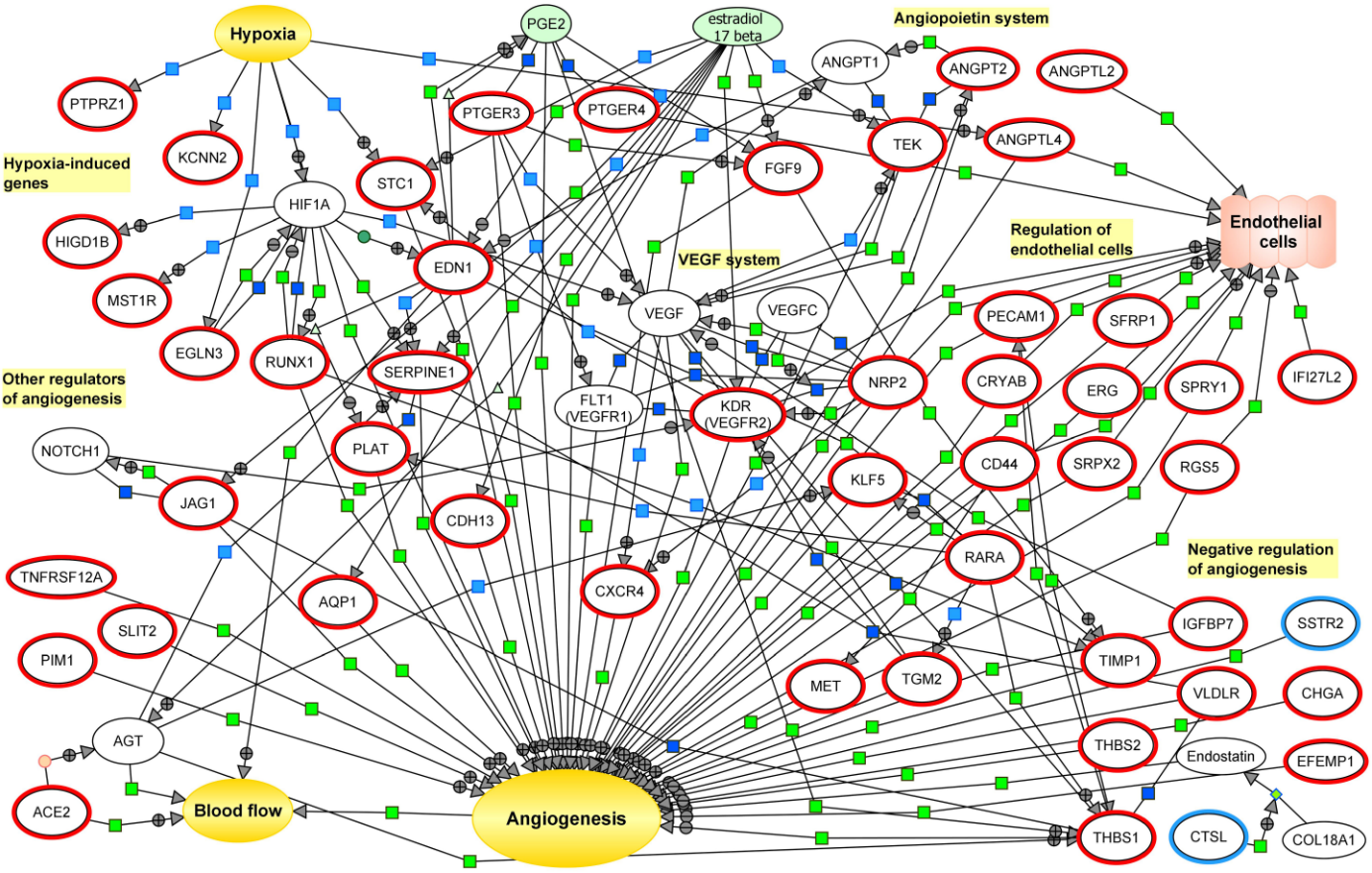

FIG. 2. Interaction network of genes related to the process of angiogenesis. Genes with higher mRNA levels in pregnant endometrium are highlighted in red, genes with lower levels in blue. Genes/proteins are in white, small molecules in green, and biological processes in yellow. Interaction types: dark blue squares: binding; light blue squares: expression; green squares: regulation; green circles: promoter binding; cyan triangles: transport; cyan diamonds: metabolism. Further information on nodes and interactions can be found in Supplement to Figure 2 (navigable HTML).

DISCUSSION

Biological Model and Quantitative Stereological Analysis of the Biopsy Samples

In order to reduce biological noise due to genetic variability in our biological model, pregnant and nonpregnant samples were obtained from the same mare (paired design) so that each animal served as its own control. The heatmap in Figure 1a demonstrates the relevance of genetic variability between animals by grouping the corresponding pregnant and control sample of each mare, thus confirming the importance of paired analysis. Potential effects by the order of sampling were excluded by randomization, i.e., for some animals the pregnant samples and for other animals the nonpregnant samples were taken first.

Because the endometrial tissue is composed of different cell types, such as surface epithelium, glandular epithelium, stromal cells, and blood vessels, all biopsy samples were analyzed by

TABLE 4. Differentially expressed genes involved in prostaglandin signaling and metabolism.

Eca gene symbol	Eca gene name	Eca Entrez gene ID	Hsa gene symbol	Hsa gene name	Hsa Entrez gene ID	FC Pr/Co[a]	q-value (%)
LOC100053557	similar to protaglandin receptor EP3E	100053557	*PTGER3*	prostaglandin E receptor 3 (subtype EP3)	5733	1.8	1.6
LOC100053208	similar to prostaglandin E2 receptor EP4 subtype	100053208	*PTGER4*	prostaglandin E receptor 4 (subtype EP4)	5734	2.0	0
LOC100070491	similar to prostaglandin F synthase	100070491	*AKR1CL1*	aldo-keto reductase family 1, member C-like 1	340811	2.3	1.3
LOC100070501	similar to prostaglandin F synthase	100070501	*AKR1CL1*	aldo-keto reductase family 1, member C-like 1	340811	2.2	2.1
PLA2G1B	phospholipase A2, group IB (pancreas)	100033889	*PLA2G4A*	phospholipase A2, group IVA (cytosolic, calcium-dependent)	5321	1.6	0.6
LOC100065438	hypothetical LOC100065438	100065438	*SLCO2A1*	solute carrier organic anion transporter family, member 2A1 (prostaglandin transporter)	6578	2.0	2.1
	ENSECAG00000004698		*PTGR1*	prostaglandin reductase 1	22949	2.7	1.8

[a] FC, fold change; Co, control; Pr, pregnant.

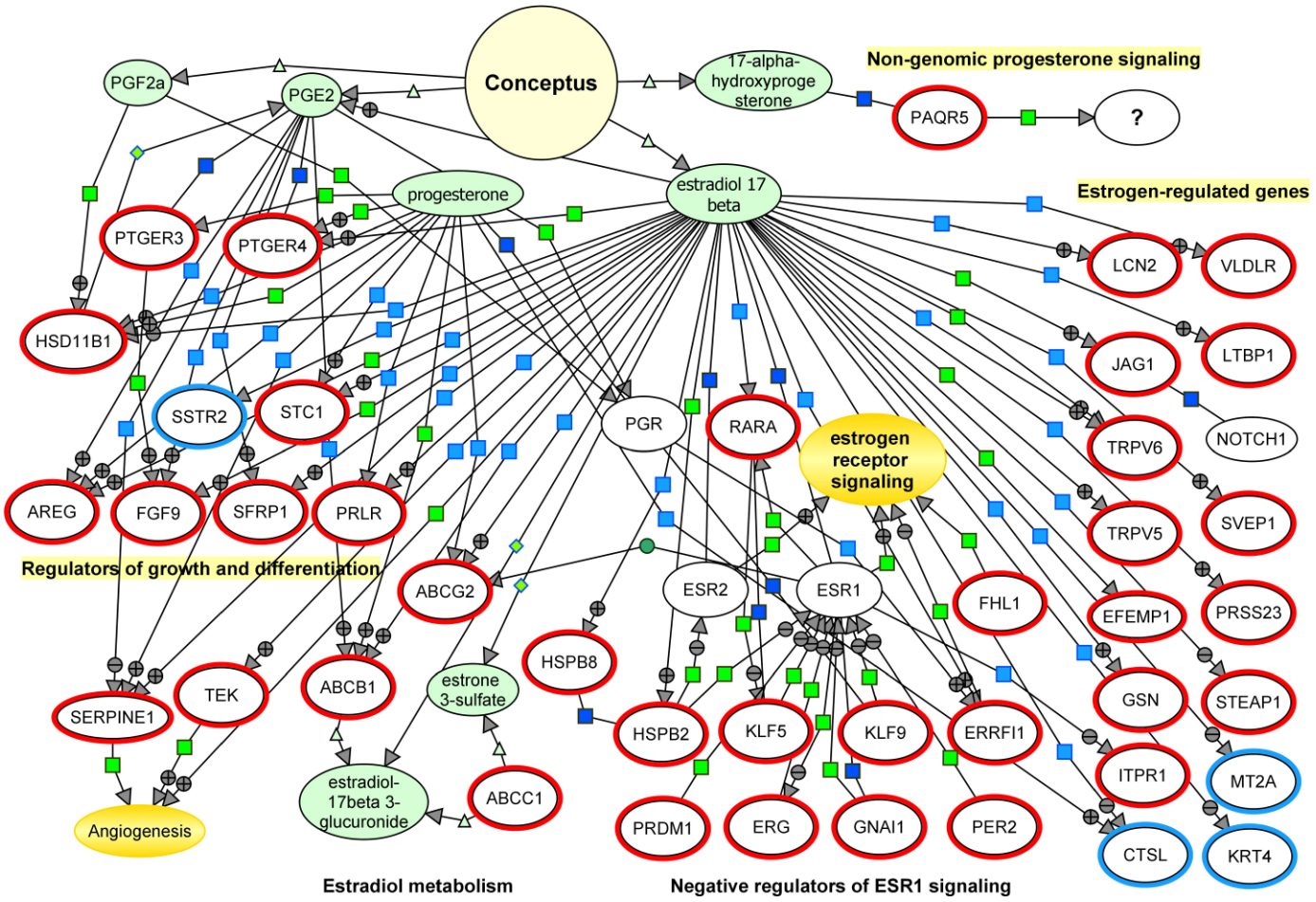

FIG. 3. Interaction network of genes related to steroid hormone and prostaglandin signaling. Genes with higher mRNA levels in pregnant endometrium are highlighted in red, genes with lower levels in blue. Genes/proteins are in white, small molecules in green, and biological processes in yellow. Interaction types: dark blue squares: binding; light blue squares: expression; green squares: regulation; green circles: promoter binding; cyan triangles: transport; cyan diamonds: metabolism. Further information on nodes and interactions can be found in Supplement to Figure 3 (navigable HTML).

quantitative stereological analysis to determine quantitative information about their tissue composition. Overall, tissue composition was very consistent within the biopsy samples. This is an important feature because biopsy sample composition can have a strong influence on microarray findings because of different mRNA concentration changes in different cell types. The 5.6-fold higher proportion of luminal epithelial cells in the Day 12 control sample of mare #3 indicated a different biopsy sample composition, most probably causing the lower or even inverse gene expression differences observed for many of the DEGs for this mare (Supplemental Table S2 and Fig. 1c). This finding underlines the importance to verify similar biopsy sample composition because this may have a strong influence on microarray results.

Differential Gene Expression at Days 8 and 12 and Between Days 8 and 12 of the Estrous Cycle

Microarray analysis of endometrial biopsy samples collected from Day 8 pregnant mares in comparison to corresponding control samples did not reveal any DEGs. Also, the exclusion of data from mare #3 because of an aberrant up-regulation of immune response genes in the pregnant sample did not result in identification of DEGs. Validation of 13 selected genes by qPCR confirmed the microarray data for these genes. This result suggests that there are no detectable changes in mRNA concentrations in endometrial biopsy samples on Day 8 of pregnancy in response to the early conceptus, which is in line with the beginning secretion of appreciable amounts of steroid metabolites by the equine embryo at around Day 10 of gestation [39, 40].

In contrast, significant expression differences were observed at Day 12 of pregnancy. For these genes, gene expression was also compared between the control samples of Days 8 and 12 and between pregnant samples of Days 8 and 12. Although the microarray analyses of Days 8 and 12 were performed at different times and slight technical biases influencing comparability of Day 8 and Day 12 data sets cannot be excluded, the results of the qPCR validation showed good agreement with the array results. The additional analysis of the expression between Day 8 and Day 12 control samples showed that most of the Day 12 (pregnant vs. control) down-regulated genes are down-regulated from Day 8 to Day 12 in the control samples as well, indicating an enhancement of down-regulation of these genes at Day 12 by the presence of a conceptus. Some of the genes up-regulated at Day 12 of pregnancy are also down-regulated from Day 8 to Day 12 in the control samples, i.e., the higher mRNA levels in Day 12 pregnant compared to Day 12 control samples are rather due to a prevention of down-regulation in response to the conceptus except for *FGF9* and *FGF9*-antisense transcripts, which are additionally up-regulated in Day 12 pregnant samples. Finally, an increased

expression from Day 8 to Day 12 in the control samples is further enhanced by the presence of a conceptus for some genes. These relatively complicated expression changes may be caused by the complex interactions of steroid hormone regulations in the equine endometrium.

Characterization of the DEGs by GSEA

GSEA revealed a number of enriched gene sets that provided a first characterization of the obtained DEGs and helped to identify genes that could have conserved functions across species. Overall, the number of genes overlapping with the top 500 genes of the ranked Day 12 gene list that contain the up-regulated genes was rather low for most of the identified gene sets. The gene set with the highest enrichment score was derived from the recently published study by Klein et al. of Day 13.5 pregnant endometrium in comparison to nonpregnant endometrium [30]. Similar to our results, more genes with higher expression levels in pregnant endometrium were found in this study. The overlap of the Day 13.5 up-regulated genes with the top 500 of our study was 24 (of 63) but only 2 for the down-regulated genes (in top 100 down-regulated genes). This could be an indication that there are different responses to the conceptus at these two time points of early pregnancy. However, comparability of the microarray results is limited because different Agilent microarrays (Klein et al. used a custom array) and different techniques (Klein et al.: dual-color hybridization and Axon scanner resulting in lower sensitivity) were used, and many of the probes on the custom array of Klein et al. are not well annotated. The significant overlap with gene sets containing genes up-regulated in human endometrium during the window of implantation [31, 41] indicates that there are similarities in gene expression changes in equine und human endometrium during early pregnancy. Furthermore, significant enrichment was found for genes induced at Day 14 of early pregnancy in porcine endometrium [38] and at Day 18 of early pregnancy in bovine endometrium (our unpublished data), but the number of genes overlapping with the top 500 of the Day 12 ranked gene list was relatively low. Higher numbers of overlapping genes with the top 500 were found for genes regulated during the estrous cycle in bovine endometrium and estrogen-induced genes in general. The gene set with the highest overlap with the top 500 genes (Boquest $CD31^+$ vs. $CD31^-$ [32]) comprised genes differentially expressed between two types of CD45 $(PTPRC)^-$ $CD34^+$ CD105 $(endoglin)^+$ stromal stem cells distinguished by the expression of CD31 (PECAM1). At first glance, the relatively high overlap with this gene set seems somewhat unexpected but can be explained by the different cell types present in the endometrium. For example, bovine endometrial stromal cells have been characterized to have similarities to mesenchymal progenitor cells [42]. Furthermore, the mRNA coding for CD31 (PECAM1), a marker of endothelial cells that has also been described in context of angiogenesis [43], was found as 1.6-fold up-regulated in the samples of Day 12 pregnant endometrium. Boquest et al. [32] described the $CD31^+$ cells as closely related to microvascular endothelial cells based on their up-regulated transcripts, which agrees well with the results of DAVID and CoPub where terms related to angiogenesis were found as quantitatively enriched. A substantial overlap was also found for the $CD31^+$ down-regulated gene set (38 genes in the top 500) that contains transcripts associated with extracellular matrix, transcripts that have been shown as expressed in early osteoblast differentiation, osteoclast-related transcripts, and transcripts typical of neuronal tissue [32]. Again, related terms were found with DAVID and CoPub, such as extracellular region, tissue remodeling, bone remodeling, neurogenesis, and inflammation. Overall, the identification of biologically very different gene sets could reflect 1) differential gene expression in different compartments of the endometrium and 2) a response to different embryonic signals. This corresponds to the fact that the equine conceptus produces different molecules [19], such as progesterone, E2, and prostaglandins.

Genes with Conserved Roles Across Species

The analysis of the endometrium-related gene sets from different species revealed a number of genes that could have conserved regulatory roles in the endometrium across species. Stanniocalcin 1 (*STC1*) has been described in multiple species, e.g., as a marker for implantation in pigs [44]. In sheep, *STC1* mRNA and protein are up-regulated in the uterine glands after Day 16 of pregnancy, probably regulating growth and differentiation of the fetus and placenta [45]. Increase of *STC1* expression has also been shown in rat uterus during embryo implantation and decidualization [46] and during the window of implantation in human endometrium [31]. In our gene expression study of bovine endometrium during the estrous cycle, highest expression levels were found at estrus, suggesting an up-regulation by E2 [47]. Crystallin, alpha B (*CRYAB*), coding for a member of the small heat shock protein (HSP20) family, is also up-regulated in human endometrium during the window of implantation [31, 41] and in bovine endometrium at Day 18 of pregnancy, as well as at estrus compared to diestrus (our unpublished data). In human myometrium CRYAB interacts with HSP27 (HSPB) and decreased CRYAB expression at the time of labor is thought to liberate HSP27 (HSPB) that participates in cytoskeletal remodeling in myometrial cells [48]. Up-regulation of *IGFBP2* was also found in porcine endometrium at Day 14 of pregnancy [38] and at Day 18 of pregnancy [49] as well as at estrus in bovine endometrium [47]. IGFBP2 expression has also been shown to be regulated by E2 and progesterone in human endometrial stromal cells [50]. Furthermore, *IGFBP1* has been reported as a common endometrial marker of conceptus elongation in sheep and cattle [51] and to mediate progesterone-induced decidualization in human endometrium [52]. In addition, IGFBP1 and TIMP metallopeptidase inhibitor 1 (TIMP1) have been demonstrated to inhibit trophoblast invasiveness in human endometrium [53, 54]. Tumor necrosis factor (ligand) superfamily member 10 (*TNFSF10*, *TRAIL*) mRNA has been shown to be up-regulated in human endometrium during the window of implantation [31] and in bovine endometrium at Day 18 of pregnancy [55]. Furthermore, a role of *TNFSF10* in the modulation of the cytokine milieu at the implantation site has been suggested based on the differential regulation of cytokines and chemokines in human endometrial stromal cells by TNFSF10 [56]. In addition to the genes at the top of Supplemental Table S4, a literature search revealed further genes described in the context of pregnancy in other species. Namely, amphiregulin (*AREG*), a member of the epidermal growth factor family, has been attributed a function in embryonic attachment in humans [53]. Abundant expression of insulin-like growth factor binding protein 7 (*IGFBP7*) has been found in human glandular epithelial cells during the secretory phase, and an in vitro knockdown revealed a role of IGFBP7 protein in differentiation of these cells [57]. In porcine endometrium induction of prolactin receptor (*PRLR*) mRNA by estradiol was shown, whereas coadministration of progesterone abolished this effect [58]. Expression of the PGE2 receptors *PTGER3* and *PTGER4* was investigated in the mouse uterus, and the observed

expression patterns in the preimplantation and postimplantation period indicated a role in uterine preparation for implantation and in the process of decidualization, respectively [59]. Moreover, a number of genes (e.g., *STC1*, *ATP2A3*, *TRPV5*, *TRPV6*) have been described in the context of calcium ion binding and regulation of calcium homeostasis that has been implicated in establishment and maintenance of pregnancy in pigs [60]. Finally, genes are up-regulated at Day 12 of pregnancy in equine endometrium that have been described as essential for successful pregnancy in the mouse, such as ERBB receptor feedback inhibitor 1 (*ERRFI1*) [61], a negative regulator of *ESR1* and nuclear receptor subfamily 2, group F, member 2 (*NR2F2*, *COUP-TFII*) [62–64]. *NR2F2* has been shown to repress the oxytocin gene promoter in human uterine epithelial cells [65] and to regulate stromal cell differentiation (decidualization) and, indirectly, the suppression of estrogen activity required for establishing a receptive uterus in the mouse [63]. In bovine endometrium we found increased expression at Day 18 of pregnancy [55] and decreased *NR2F2* transcript levels in endometrium from clone pregnancies vs. IVF pregnancies at Day 18 of pregnancy [66].

Genes Related to Angiogenesis and Vascular Remodeling

The search for quantitatively enriched functional terms (DAVID) and biological keywords (CoPub) associated with the Day 12 up-regulated genes revealed the highly enriched functional term angiogenesis. In the context of this process, increased endometrial vascular perfusion has been shown on Days 12–16 in both uterine horns of pregnant mares compared to nonpregnant mares by transrectal color Doppler ultrasonography [67]. Also, dysregulation of angiogenesis in the endometrium during early pregnancy has been found in the context of pregnancy failure [68]. To get an overview of the angiogenesis-related genes represented in the DEGs and their putative interactions, an interaction network was drawn (Fig. 2). DEGs were found for many regulatory systems of the complex process of angiogenesis, namely the VEGF system (receptors *KDR*, *NRP2*), the angiopoietin family (*ANGPT2*, *ANGPTL2*, *ANGPTL4*, *TEK*), different regulators of endothelial cells, and hypoxia-induced genes. There are also negative regulators of angiogenesis up-regulated in Day 12 pregnant endometrium, such as thrombospondins 1 and 2 (*THBS1*, *THBS2*), known inhibitors of endothelial cells and angiogenesis [69]. The complex regulation of angiogenesis and the results of the quantitative stereology (no difference in the proportion of blood vessels between pregnant and control samples) indicate that there is a remodeling of vascularization rather than neoangiogenesis or that neoangiogenesis is not yet microscopically detectable in Day 12 pregnant endometrium. This remodeling of vascularization is likely to play a role in maternal support of conceptus growth and in preparing the uterus for the prospective pregnancy.

Genes Related to Steroid Hormone and Prostaglandin Signaling

Furthermore, many genes were found that are probably regulated by the steroid hormones E2 and progesterone in Day 12 pregnant endometrium. This is in line with the finding that the embryo begins to secrete significant amounts of estrogens as early as Day 10 after ovulation [70, 71] and progesterone is the key hormone that prepares the endometrium for establishment and maintenance of pregnancy [72]. Conceptus estrogens are also supposed to have multiple effects on early pregnancy, such as stimulation of early conceptus migration and changes in uterine tonicity, blood flow, and endometrial secretory activity important to the nutrition of the preimplantation conceptus [73]. An important mediator of estrogen signaling in equine endometrium could be *FGF9* (microarray 9-fold, qPCR 8-fold up-regulated in Day 12 pregnant endometrium) that has been described as an autocrine endometrial stromal growth factor induced by E2 in human endometrial stroma [74]. Induction of *FGF9* expression by PGE2 through the EP3 receptor was also demonstrated in human endometrium [75]. In contrast to the localization in human endometrium, FGF9 protein expression in the porcine endometrium has been detected in the glandular epithelium at Day 14 of pregnancy [38]. The complex expression pattern of *FGF9* mRNA (see above) and the up-regulation of a putative antisense transcript (8-fold, Supplemental Table S2) make this gene an especially interesting candidate.

In addition to genes up-regulated by E2, a number of negative regulators of estrogen signaling, e.g., *KLF5*, *ERRFI1*, and *HSPB2* (Fig. 3), were found as up-regulated that could be indications for either a negative feedback regulation in response to the E2 signal or the result of progesterone action on the endometrium. A study of steroid metabolites produced by the equine conceptus revealed 17-alpha-OH-progesterone as the major steroid metabolite [39]. Interestingly, this metabolite binds to the progestin and adipoQ receptor family member V (PAQR5) [76], also known as membrane progestin receptor gamma, which is up-regulated in Day 12 pregnant endometrium (qPCR: 4.7-fold). PAQR5 is one of the receptors mediating nongenomic effects of progesterone. The equine conceptus is also known to secrete prostaglandins E2 and F2-alpha [12] that could play a role in pregnancy recognition and prevention of luteolysis. A number of genes that function in context of prostaglandin signaling and metabolism were found as up-regulated. Furthermore, mRNAs of PGE2 receptors EP3 (*PTGER3*) and EP4 (*PTGER4*) were up-regulated, similar to findings in the pig, in which *PTGER2* is up-regulated in early pregnancy [77]. However, in contrast to studies in porcine endometrium, mRNA levels of prostaglandin E synthases did not differ between pregnant and nonpregnant equine endometrium. There was also no difference in mRNA levels for the known $PGF_{2\alpha}$ synthases; only two predicted $PGF_{2\alpha}$ synthases that have homology to *AKR1CL1* (pseudogene in humans) were approximately 2-fold up-regulated. Unlike in ruminants, where up-regulation of mRNA for oxytocin receptor (*OXTR*) is prevented by the signaling of interferon tau [78], *OXTR* mRNA was slightly up-regulated in equine endometrium at Day 12 of pregnancy.

Genes Possibly Related to the Process of Mechanotransduction

Although the results of this study suggest an endometrial response to different signaling molecules, this does not exclude a mechanical signaling induced by the migrating conceptus. In a recent study a small intrauterine device (water-filled plastic ball with a diameter of 20 mm) was shown to induce prolonged luteal function [21], further supporting the concept of pregnancy recognition via mechanosensation. A study in sheep also described changes at the maternal-conceptus interface and uterine wall during pregnancy reflecting an increased mechanosensation and mechanotransduction [79]. Possibly, changes in mRNA expression levels at Day 12 of pregnancy in the mare could in part reflect mechanosensation responses to the conceptus. Some of the up-regulated genes of our study were already described in the context of mechanotransduction: a direct response to mechanical force has been shown for PECAM1 protein [80]; up-regulation of IGFBP1 secretion in

response to mechanical stretch was found by Harada et al. [81] in decidualized endometrial stromal cells; two members (*RND1*, *RND3*) of the Rho GTPase family (key regulators of cytoskeletal signaling) and a Rho GTPase activating protein (*ARHGAP29*) are up-regulated in Day 12 pregnant endometrium; and Rho activation has been described in the context of mechanotransduction-associated alveolar epithelial cell differentiation [82].

In conclusion, this study is the first systematic analysis of maternal transcriptome changes in response to the presence of an embryo in the mare on Days 8 and 12 of pregnancy. The stereological analysis of the biopsy samples showed that the homogenous composition of endometrial biopsies is an important issue for endometrial transcriptome analysis. No changes in endometrial gene expression were detectable at Day 8 of pregnancy. The DEGs identified on Day 12 in response to the early embryo evidence the orchestrated roles of estrogens, progesterone, and prostaglandin E2 in regulating gene expression in the equine endometrium in context of establishment and maintenance of pregnancy. Additionally, a form of mechanotransduction by the migrating conceptus is likely of importance. A large number of interesting candidate genes and biological processes were identified as potentially important for endometrial remodeling in response to the early embryo and need further detailed analysis.

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