Differential Endometrial Gene Expression in Pregnant and Nonpregnant Sows

Authors: Esben Østrup, Stefan Bauersachs, Helmut Blum, Eckhard Wolf, and Poul Hyttel
Source: Biology of Reproduction, 83(2) : 277-285
Published By: Society for the Study of Reproduction
URL: https://doi.org/10.1095/biolreprod.109.082321
Differential Endometrial Gene Expression in Pregnant and Nonpregnant Sows

Esben Østrup, Stefan Bauersachs, Helmut Blum, Eckhard Wolf, and Poul Hyttel

Department of Basic Animal and Veterinary Sciences, Faculty of Life Sciences, University of Copenhagen, Frederiksborg, Denmark

Laboratory for Functional Genome Analysis (LAFUGA) and Chair for Molecular Animal Breeding and Biotechnology, Gene Center, Ludwig-Maximilians University of Munich, Munich, Germany

ABSTRACT

In an attempt to unveil molecular processes controlling the porcine placentation, we have investigated the pregnancy-induced gene expression in the endometrium using the Affymetrix GeneChip Porcine Genome Array. At Day 14 after insemination, at the time of initial placentation, samples were obtained from the endometrium of pregnant sows and sows inseminated with inactivated semen. Analysis of the microarray data revealed 263 genes to be significantly differentially expressed between the pregnant and nonpregnant sows. Most gene ontology terms significantly enriched at pregnancy had allocated more up-regulated genes than down-regulated genes. These terms included developmental process, transporter activity, calcium ion binding, apoptosis, cell motility, enzyme-linked receptor protein signaling pathway, positive regulation of cell proliferation, ion homeostasis, and hormone activity. Only the three terms oxidoreductase activity, lipid metabolic process, and organic acid metabolic process had an overrepresentation of down-regulated genes. A gene interaction network based on the genes identified in the gene ontology term developmental process identified genes likely to be involved in the process of placentation. Pregnancy-specific localization of IL11RA to the surface epithelium of the endometrium suggests a role of interleukin 11 signaling in formation of the porcine epitheliochorial placenta. Furthermore, up-regulation of FGF9 mRNA in pregnant endometrium and localization of FGF9 to the apical cell domain of the glandular epithelium suggest the concept of endometrial FGF9 acting as an embryonic growth factor in the pig.

endometrium, ERBB3, FGF9, FGF3, IL6R, IL11RA, implantation, LIFR, MUC4, placenta, placentation, pregnancy, uterus

INTRODUCTION

Successful embryonic development is dependent on a complex molecular cross-talk between the embryo(s) and the maternal organism [1, 2]. In the pig, the embryo-maternal communication becomes evident at Day 12 of gestation, when the conceptuses release a surge of estrogen [3]. This embryonic signal for maternal recognition of pregnancy triggers changes in the prostaglandin metabolism of the endometrium to prevent regression of the corpora lutea by prostaglandin F2α (PGF2α) [4]. Recent research indicates that the estrogen signal from the conceptuses stimulates endometrial prostaglandin E2 (PGE2) synthesis. Combined with a positive PGE2 feedback loop in the endometrium, this synthesis leads to an increase in the PGE2:PGF2α ratio, which helps to overcome the luteolytic effect of PGF2α [5].

Following the embryonic signal for maternal recognition of pregnancy at Day 12, the porcine embryos remain free-floating in the uterine lumen until Days 13–14 of gestation, when they appose and subsequently attach to the uterine luminal epithelium [6]. Until this time of development, the embryos are supported from the endometrium by histiotrophic nutrition. From Days 15–20 of gestation, a gradual transition in embryonic nutrient takes place from being mainly histiotrophic to becoming primarily hemotrophic [6]. The functional changes are accomplished by extensive tissue remodeling of the endometrium [7, 8], where a pronounced vascularization is evident already from Day 13 of gestation [9, 10]. These processes, together with the attachment of the embryos to the surface epithelium of the uterus, initiate the placentation. The porcine placenta is, in contrast to that of many other species, epitheliochorial [11, 12]. That is, the placentation is noninvasive, and the placental barrier includes both the trophoblast and the epithelium of the endometrium.

During evolution, several subtypes of placentas have developed in eutherian mammals [11, 13]. However, the basic principles have not changed. Similar to all other subtypes of chorio-allaantoic placentae, the epitheliochorial placenta is essential for the growth and development of the embryo and fetus. Despite the evolutionary changes in placental architecture, strong similarities also exist in the cellular functions between different placental subtypes [13]. Microarray investigations have been conducted successfully in several species in an attempt to disclose the mechanisms of placentation [14–16]. A comparison of similar studies, however, reveals large differences in the gene expression profile between the species [16], indicating a highly species-specific molecular regulation of placentation. Differences in the embryo maternal communication are not necessarily related to differences in the placental structure. In sheep and cows, the signal for maternal recognition of pregnancy is interferon tau, produced by the conceptuses [17]. The porcine conceptuses also produce interferons; however, they do not appear to be antiluteolytic [18]. Instead, it has been suggested that interferon gamma, the main interferon produced by the porcine conceptuses, might be important in regulation of angiogenesis in the endometrium, similar to the human and murine placenta [19]. However, the

1Supported by the Danish Pig Production, the Danish Agency for Science, Technology and Innovation grant 09-060623/FTP, and the German Federal Ministry for Education and Research (BMBF, FUGA-TOplus Compendium). Microarray data have been submitted to the NCBI Gene Expression Omnibus with accession no. GSE18641.

2Correspondence: Esben Østrup, Department of Basic Animal and Veterinary Sciences, Faculty of Life Sciences, University of Copenhagen, Grønnehøjvej 7, 1870 Frederiksberg C, Denmark. FAX: 0045 353 32547; e-mail: esostrup@gmail.com
pregnancy-specific role(s) of estrogen- and interferon-stimu-
lated genes in the porcine endometrium remains largely 
differentially expressed at gestation, have been investigated 
[18]. In the present study, we aim to investigate pregnancy-
induced changes in the gene expression of the porcine 
endometrium in an attempt to identify specific mechanisms 
involved in the regulation of the initial porcine placenta-
tion.

MATERIALS AND METHODS

Animals

Three pairs of Danish Landrace and three pairs of Yorkshire sows, each pair from the same litter, were inseminated and slaughtered in pairs. One sow in each pair was inseminated with a standard dose of single Duroc semen, whereas the other littermate was inseminated with a dose of freeze-inactivated semen from the same boar. The animals were slaughtered at Day 14 postinsemination. The uteri were removed, and each uterine horn was flushed with PBS containing 1% fetal calf serum and subsequently opened longitudinally at the antimesometrial side. The sites of embryonic attachment were macroscopically 
visual in the endometrium on the mesometrial side in the form of hyperemic 
zones (Fig. 1). In pregnant sows, samples of the endometrium (lamina 
epithelialis, lamina propria, and tela submucosa, but not tunica muscularis) 
were taken from such hyperemic zones at three locations of each uterine horn:
proximal (the end close to the ovaries), intermedial, and distal (next to the 
corpus uteri). Samples were taken from the endometrium of the nonpregnant 
corpus uteri). Samples were taken from the endometrium of the nonpregnant 
animals at comparable locations. Tissue samples were transferred to RNAlater 
(Ambion) or 4% paraformaldehyde (PFA)/PBS within 25 min after slaughter. 
Samples in RNAlater were incubated overnight at 4°C and stored at −80°C until 
forward processing. Samples in RNAlater were incubated overnight at 4°C and stored at −80°C until further processing. Samples in 4% PFA/PBS were incubated overnight at 4°C and stored in 1% PFA/PBS at 4°C until embedding in paraffin. All procedures 
involved animals were conducted in accordance with the national guidelines 
for agricultural animal care.

Microarray Analysis

Total RNA was isolated from endometrium using TRIzol (Invitrogen) 
according to the manufacturer’s recommendations. An additional RNA 
selective precipitation was made using ammonium acetate. Purity and quantity 
of total RNA was measured by use of Nanodrop ND-1000 spectrophotometer 
(Thermo Fisher Scientific), and quality was assessed by electrophoresis. RNA 
samples were tested for genomic DNA contamination by performing a 
quantitative RT-PCR without reverse transcriptase in the RT step.

Equal amounts of total RNA from samples of proximal, intermedial, and 
distal endometrial sections were pooled for each animal. Probe synthesis and 
hybridization to the porcine GeneChip from Affymetrix were performed 
according to the recommendations of the manufacturer. Cel files were 
processed using the Robust Multi-Array normalization in the BioConductor 
package affy for R [20]. Microarray data have been deposited in the National 
Center for Biotechnology Information’s Gene Expression Omnibus [21] and 
are accessible through GEO Series accession number GSE18641 (http://www.
cbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18641). For quality control, nor-
amalized data were analyzed with a distance matrix and a heatmap based on 
pair-wise distances (BioConductor package geneplotter). Two samples were 
excluded from further analysis. Significance analysis was performed using the 
BioConductor package Linear Models for Microarray Data (LIMMA) [22].

Furthermore, DAVID was used to calculate the fold-enrichment of 
identified Gene Ontology (GO) terms. The fold-enrichment for a particular 
GO term describes the ratio between the numbers of genes in the gene list 
belonging to a specific GO term and the total number of genes in the gene list, 
which have at least one GO annotation. This ratio is then compared to the ratio 
between the total number of genes in the GO term and the total number of 
genes in the human genome with at least one GO annotation. For example, if 8 
(3.5%) of 223 genes in the gene list are involved in hormone activity and the 
figure in the human genome is 127 (0.75%) of 16968 genes associating with 
hormone activity, the fold-enrichment is roughly 3.5% /0.75% ≈ 4.7. Fold- 
enrichment for the remaining GO terms was calculated in a similar manner.

An interaction network was built using Pathway Architect Software 
(version 2.0.1; Stratagene). Interactions provided by the Pathway Architect 
databases were cross-checked with literature, and additional interactions 
were assigned to the network. The human Entrez Gene IDs of the putative human 
orthologous genes for the identified porcine transcripts were used for these 
analyses.

Quantitative Real-Time RT-PCR

The same RNA as used for the microarrays was used to validate the results 
by real-time RT-PCR (see Table 4). One microgram of total RNA was reverse 
transcribed in a total volume of 25 μl, containing 1× buffer (Promega), 0.5 mM

![FIG. 1. Hyperemic zone in the endometrium identifying the sites of implantation, as seen after uterine flushing.](image-url)
dNTPs (Fermentas), 16 ng/μL of random hexamer primers (Fermentas), 8 ng/μL (μM) of oligo-dT primers (Fermentas), 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega), and 32 U of RNase H Minus, Point Mutant (Promega). Primers were designed to amplify specific fragments referring to selected regulated genes (Table 1). Amplified PCR fragments were sequenced with forward and reverse primers to verify the resulting PCR product. The specific melting point of the ampliﬁed product was determined with the LightCycler standard PCR protocol served as a veriﬁcation of the product identity in the following PCR procedures. Real-time PCR reactions were performed in a 10-μL reaction mixture (2 μL of cDNA, 1 μM forward and reverse primer, and 1× Light Cycler DNA Master SYBR Green I [Roche]) using a real-time LightCycler 480 instrument (Roche). The annealing temperature was 60°C for all reactions. A no-template control was made in all runs, using water instead of cDNA.

The reference gene ubiquitin B (UBB), which previously has been used as a reference gene in the endometrium [16], showed no statistical difference between pregnant and nonpregnant animals according to the microarray data verifying its suitability as reference gene. Expression levels of the selected target genes were calculated using relative quantitation (LightCycler 480 software version 1.5.39). The data were normalized to the expression of UBB, and a calibrator was included to normalize between runs. Five biological and three technical replicates were made for all quantitative PCR reactions. Calculations of signiﬁcant difference in the real-time RT-PCR expression data between samples from pregnant and nonpregnant animals were carried out in R using Wilcoxon signed-rank tests.

Immunohistochemistry

Endometrial samples stored in 1% PFA/PBS were dehydrated and embedded in parafﬁn. Parafﬁn sections (thickness, 5 μm) mounted on Superfrost Plus slides (Gerhard Menzel, Braunschweig, Germany) were dewaxed followed by blocking of endogenous peroxidase with 0.5% (v/v) H2O2 in 99% ETOH. After rehydration, sections were submitted to heat-induced epitope retrieval by microwave treatment in either 0.01 M sodium citrate buffer (pH 6.0) or in ethylenediaminetetra-acetic acid at either pH 8 or pH 9 (Table 2). Nonspeciﬁc binding of antibodies was blocked with PBS containing 1.25% normal horse serum (Vector Laboratories) and 1% bovine serum albumin (Sigma-Aldrich). Incubation with the primary antibodies was performed at room temperature for 2 h or overnight at 4°C (see Table 2 for details on speciﬁc antibodies). The antibodies against ERBB3 and IL6R were preadsorbed with Poly-L-lysine (Sigma-Aldrich) overnight at 4°C to reduce unspeciﬁc binding [27, 28]. Controls were made using preadsorption controls for all polyclonal antibodies using the speciﬁc blocking peptides IL6R (Santa Cruz), IL11RA (Santa Cruz), ERBB3 (Santa Cruz), LIFR (R&D Systems), and isotype controls for monoclonal antibodies using mouse IgG2a and IgG3 (Abcam). Detection of the primary antibodies was performed using ImmPRESS (Vector Laboratories). The ImmPRESS secondary antibodies were preadsorbed with 0.1% normal swine serum (DAKO) at 4°C overnight to minimize background. The color reaction was developed with an AEC-kit (Zymed Laboratories). Sections were counterstained with Mayer hematoxylin before mounting in Glycergel (Dako).

RESULTS

Microarray Analysis

A heatmap analysis of the normalized microarray data (data not shown) revealed one pregnant and one nonpregnant animal as having highly abnormal gene expression when compared to the expression-pattern of the remaining 10 animals. One of these two animals presented signs of weak endometritis at the time of sample collections. The data from these two animals were not included in the further analyses.

When comparing the gene expression in the endometrium of pregnant sows (n = 5) with that of nonpregnant sows (n = 5), 323 probe sets were identiﬁed to be more than twofold signiﬁcantly differentially expressed (Supplemental Tables S1 and S2 available at www.biolreprod.org). Annotation of the 323 probe sets revealed 146 genes expressed at higher levels in pregnant animals (referred to hereafter as up-regulated genes). Another 117 genes were detected with lower expression in the pregnant animals (referred to hereafter as down-regulated genes). From 18 probe sets, which could not be annotated, 13 were up-regulated, and ﬁve were down-regulated.

The 263 genes identiﬁed to be differentially expressed were analyzed using the functional annotation chart and the functional annotation clustering tool from DAVID [26]. The analyses were based on the major categories Biological Process and Molecular Function. A combination of the most informative signiﬁcantly enriched GO terms from the resulting functional clusters and GO terms that were not included in the functional clustering but were found to be signiﬁcantly enriched by the functional annotation chart have been summarized in Table 3. Of the GO terms that were signiﬁcantly enriched, most terms had allocated more up-regulated genes than down-regulated genes at pregnancy. Among these were GO terms such as developmental process, apoptosis, cell motility, and positive regulation of cell proliferation, which are likely to be enriched because of remodeling of the endometrium in relation to the placentaion. Another group of enriched GO terms contain genes involved in formation and regulation of histiotrophe (e.g., transporter activity, calcium ion binding, and ion homeostasis). Furthermore, GO terms describing different aspects of communication and signaling, response to external stimulus, enzyme-linked receptor protein signaling pathway, and hormone activity were present in the group of signiﬁcantly enriched GO terms, with an over-representation of up-regulated genes. Only three GO terms had an overrepresentation of down-regulated genes. These GO terms were oxidoreductase activity, lipid metabolic process, and organic acid metabolic process. The enriched terms with most down-regulated genes included genes involved in steroid hormone and prostaglandin metabolism.

Using Pathway Architect software (version 2.0.1; Stratagene), an interaction network was built based on the genes present in the GO term developmental process (Fig. 2). This network includes possible interactions between genes involved in cytokine and growth factor-mediated cell signaling. Among the genes identified in our results contributing to the cytokine-mediated signaling are genes related to the interleukin families.

### Table 2. Primary antibodies, HIER treatment, and incubation time.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Manufacturer</th>
<th>Product code</th>
<th>Source</th>
<th>Clonality</th>
<th>Isotype</th>
<th>Concentration (μg/ml)</th>
<th>HIER*</th>
<th>Primary antibody incubation*b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human LIFR</td>
<td>R&amp;D Systems</td>
<td>AF-249-NA</td>
<td>Goat</td>
<td>Polyclonal</td>
<td>IgG</td>
<td>0.50</td>
<td>Ph 9</td>
<td>2 h RT</td>
</tr>
<tr>
<td>Anti-human IL6R</td>
<td>R&amp;D Systems</td>
<td>sc-993</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>IgG</td>
<td>0.10</td>
<td>Ph 6</td>
<td>2 h RT</td>
</tr>
<tr>
<td>Anti-human IL11RA</td>
<td>Santa Cruz</td>
<td>sc-8413</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>IgG3</td>
<td>20.00</td>
<td>Ph 9</td>
<td>2 h RT</td>
</tr>
<tr>
<td>Anti-human FGF9</td>
<td>Santa Cruz</td>
<td>sc-13121</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>IgG2a</td>
<td>8.00</td>
<td>Ph 9</td>
<td>2 h RT</td>
</tr>
<tr>
<td>Anti-human MUC4</td>
<td>Abnova</td>
<td>H00005485-M07</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>IgG2a</td>
<td>0.20</td>
<td>Ph 9</td>
<td>2 h RT</td>
</tr>
<tr>
<td>Anti-human ERBB3</td>
<td>Santa Cruz</td>
<td>sc-285-G</td>
<td>Goat</td>
<td>Polyclonal</td>
<td>IgG</td>
<td>0.67</td>
<td>Ph 9</td>
<td>0/4°C</td>
</tr>
</tbody>
</table>

*a HIER, heat induced epitope retrieval.
*b RT, room temperature; o/n, over night.
These include the receptors IL6R, LIFR, and IL11RA. Both IL6R and LIFR were found to be up-regulated, whereas IL11RA was down-regulated. Among the growth factor-related genes, FGF9 was up-regulated. The FGF9 receptor FGFR3 was, however, down-regulated. Another growth factor receptor, ERBB3, was identified by the microarray to be up-regulated. A possible interaction was found by the network between ERBB3 and the mucin MUC4. The mucin MUC4 was approximately ninefold higher expressed in the endometrium of the pregnant animals compared to the expression in the nonpregnant animals.

Quantitative RT-PCR

Based on the interaction network, seven genes (IL6R, LIFR, IL11RA, MUC4, ERBB3, FGF9, and FGFR3) were selected for mRNA quantification by quantitative RT-PCR (Table 4). The results obtained by array hybridization were clearly confirmed, and more accurate gene expression differences were obtained.

Immunohistochemistry

The localization of IL6R, LIFR, IL11RA, MUC4, ERBB3, FGF9, and FGFR3 proteins was investigated in the endometrial tissue (Fig. 3).

IL6R. Strong staining for IL6R was seen in surface epithelium, superficial and deep glandular epithelium, as well as stroma cells, endothelial cells, and leukocytes. No differences were observed in the localization of IL6R between pregnant and nonpregnant animals (Fig. 3, a and b).

LIFR. Staining for LIFR was present in the surface epithelium as well as in superficial and deep glandular epithelium, with a tendency for weaker staining in the deeper glands. Staining for LIFR was also seen in some stromal cells, endothelial cells, and leukocytes. No differences were observed in the protein localization of LIFR between pregnant and nonpregnant animals (Fig. 3, d and e).

IL11RA. Staining for IL11RA was found in superficial glandular epithelium and in most of the deeper glandular epithelium in both pregnant and nonpregnant animals (Fig. 3, g and h). Furthermore, IL11RA was detected in the surface epithelium of all pregnant animals, whereas it was only detected in few cells at this location in nonpregnant animals.

MUC4. Strong staining for MUC4 was observed in the surface epithelium as well as in superficial and deep glandular epithelium. In the surface epithelium, MUC4 staining was primarily cytoplasmic, whereas in the glandular epithelium, MUC4 was strongly localized to the extracellular matrix and the apical domain of the cells. No differences were seen in the localization of the protein between pregnant and nonpregnant animals (Fig. 3, j and k).
ERBB3. Staining for ERBB3 was found in the surface epithelial cells as well as in superficial glandular epithelium. Weaker staining was observed in the deeper-lying glandular epithelium. No differences were observed in the protein localization of ERBB3 between pregnant and nonpregnant animals (Fig. 3, m and n).

FGF9. Strong staining for FGF9 was present in the apical cell domain of superficial and deep glandular epithelium.
Weaker staining was observed in the surface epithelium and stromal cells. No differences in the localization of the protein were observed between pregnant and nonpregnant animals (Fig. 3, p and q).

**FGFR3.** Staining for FGFR3 was primarily localized to the glandular epithelium and, to some degree, the surface epithelium. No differences were seen in the localization of FGFR3 between pregnant and nonpregnant animals (Fig. 3, s.

---

**TABLE 3.** Significantly enriched gene ontology terms.

<table>
<thead>
<tr>
<th>Gene ontology term</th>
<th>No. of genes</th>
<th>Genes</th>
<th>Fold enrichment</th>
<th>P value for enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0032502 developmental process</td>
<td>66</td>
<td>CD24, PRKCA, FIGPB2, PTLHL, INHBA, EDN RB, IL6R, EFHD1, LYZ, CLDN11, NUPR1, SCIN, IRS2, INHBb, ERBB3, LAMB3, SH3GL2, FOSL2, MTR, TRA, HNF1B, ARSE, FGFP, FYH, ST6GAL1, PLXDC2, GB3, RFL, CDN1NC, GCLC, ENP1, GULP1, CYCS, MAL, PLGAL1, CALR, IFIH1, KLFL, FZD2, BCL6, CD14, ITGAV, EDG1, SCML2, GNPTAB</td>
<td>1.39</td>
<td>0.003</td>
</tr>
</tbody>
</table>

| GO:0005215 transporter activity    | 31           | SLC16A1, TRPV6, CANCA1B, SLC2A1, STEAP1, SLC39A2, AQP3 | 1.60 | 0.010 |

| GO:0016491 oxidoreductase activity | 27           | GPX3, KMO, AKR1B1, STEAP1, SOD3, COQ6, F5, IFI30, LTB4DH, CYCS | 2.15 | 0.000 |

| GO:0005509 calcium ion binding     | 24           | S100A9, TRPV6, CAPNS1, PRKCA, CANCA1B, PTLHL, EFHD1, SCIN, F5, ARSE, KIAA0494, CALR, PROS1, MAN1A1, PLS3, ITGAV, GNPTAB | 1.81 | 0.007 |

| GO:0006629 lipid metabolic process | 23           | SLC16A1, TRPV6, CAPNS1, PRKCA, CANCA1B, PTLHL, EFHD1, SCIN, F5, ARSE, KIAA0494, CALR, PROS1, MAN1A1, PLS3, ITGAV, GNPTAB | 2.06 | 0.002 |

| GO:0006915 apoptosis               | 22           | CD24, PRKCA, INHBA, NUPR1, SCIN, RFL, GC1C, GULP1, CYCS, MAL, PLGAL1, CALR, IFIH1, BCL6, CD14 | 1.92 | 0.005 |

| GO:0009605 response to external stimulus | 22 | S100A9, NPY, STC1, PRKCA, INHBA, LYZ, INHBb, F5, TLR4, ENP1, PROS1, BCL6, CD14, PLA2G7, LNY6, EDG1, VWF | 2.34 | <0.001 |

| GO:0006082 organic acid metabolic process | 21 | PRKCA, MTR, GCLC, LTB4DH, ASS1, CAD | 2.50 | <0.001 |

| GO:0006928 cell motility           | 14           | CD24, SEL, NPY, PRKCA, EDN RB, FYH, GAB1, FEZ2, BCL6, EDG1 | 2.28 | 0.009 |

| GO:0007167 enzyme linked receptor protein signaling pathway | 14 | PRKCA, INHBA, IRS2, ERBB3, RB7, FGF9, CDKN1C, GAB1, SORBS1, FMOD, HPGD, EG, CYRAb, FGF3 | 3.37 | 0.000 |

| GO:0008284 positive regulation of cell proliferation | 11 | CD24, CAPNS1, PTLHL, IRS2, FGF9, LIFR, BCL6, EDG1 | 3.14 | 0.003 |

| GO:0005801 ion homeostasis          | 11           | CD24, STC1, PRKCA, EDN RB, AGTR1, TFRC, GC1C, CALR, EDG1 | 3.20 | 0.002 |

| GO:0005179 hormone activity         | 8            | NPY, NMB, STC1, PTLHL, INHBA, INHBb | 4.69 | 0.002 |

a Genes identified to be significantly up- or down-regulated by the microarray; up-regulated genes are presented in bold.

---

**TABLE 4.** Validation of microarray results using real-time RT-PCR.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Relative expression (mean ± SEM)</th>
<th>Mean fold changes</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pregnant</td>
<td>Nonpregnant</td>
<td>qPCR</td>
</tr>
<tr>
<td>MUC4</td>
<td>1.66 (0.30)</td>
<td>0.08 (0.02)</td>
<td>19.7</td>
</tr>
<tr>
<td>ERBB3</td>
<td>2.09 (0.45)</td>
<td>0.55 (0.10)</td>
<td>3.8</td>
</tr>
<tr>
<td>IFIR</td>
<td>1.53 (0.17)</td>
<td>0.59 (0.04)</td>
<td>2.6</td>
</tr>
<tr>
<td>IL6R</td>
<td>2.14 (0.25)</td>
<td>0.38 (0.08)</td>
<td>5.7</td>
</tr>
<tr>
<td>IL11RA</td>
<td>2.15 (0.51)</td>
<td>4.90 (0.40)</td>
<td>–2.3</td>
</tr>
<tr>
<td>FGF9</td>
<td>1.86 (0.26)</td>
<td>0.45 (0.03)</td>
<td>4.1</td>
</tr>
<tr>
<td>FGR3</td>
<td>0.70 (0.08)</td>
<td>1.03 (0.10)</td>
<td>–1.5</td>
</tr>
</tbody>
</table>
and t); however, a tendency was found for higher staining intensity in the luminal epithelium of the pregnant animals compared to the nonpregnant.

**DISCUSSION**

In the present study, we successfully identified 263 genes that were significantly differentially expressed in the porcine endometrium between pregnant and nonpregnant sows. Based on an interaction network, we selected the genes IL6R, LIFR, IL11RA, MUC4, ERBB3, FGF9, and FGFR3, which are potential candidate genes for regulation of placentaion.

The interleukin 6 family of cytokines is well known to be a key regulator of implantation and placentaion. In mice, these cytokines as well as their receptors are required for normal implantation and embryonic survival [29]. In the present study, we identified the specific receptors IL6R, LIFR, and IL11RA to be significantly differentially expressed; however, neither the cytokines nor the common signal transducer, IL6ST, was among the differentially expressed genes. This indicates that endometrial regulation of signaling in this cytokine family is, to a great extent, controlled by the expression of the specific receptors.

The receptor IL6R was localized in both surface and glandular epithelium, as well as to cells of the stroma, in both pregnant and nonpregnant animals. This localization of the IL6R in the porcine endometrium is similar to that reported in human endometrium [30].

LIFR was similar to IL6R localized to both surface and glandular epithelium, as well as to some stromal cells of the porcine endometrium. The LIFR mRNA expression was similar to that in mouse [31], increased in pregnant animals; however, no apparent differences were noticed in the localization of IL6R between pregnant and nonpregnant animals. This localization of LIFR is similar to that reported in the endometrium of the rhesus monkey during the luteal phase and peri-implantation period [32]. In human and mouse, LIFR mRNA was shown to be exclusively expressed in the surface epithelium at the time of implantation [31, 33], and only weak staining for LIFR has been reported in human glandular epithelium [30, 34].

In contrast to the two other receptors in the IL6 cytokine family, IL11RA was significantly lower expressed in the endometrium from the pregnant animals compared to the nonpregnant (Table 4). In human endometrium, it is without any cyclic variation [35].

Interestingly, a marked increase in the number of cells stained for IL11RA protein was identified in the surface epithelium of pregnant compared to nonpregnant sows (Fig. 3), despite the down-regulation of IL11RA mRNA in the endometrial tissue samples from pregnant sows. The role of IL11 has been investigated in mice [36] and humans, in which IL11 stimulates migration of extravillous trophoblast cells but prevents their invasion [37, 38]. The crucial role of IL11 in prevention of invasion is also supported by studies in Ill1ra knock-out mice. Hence, mice lacking Ill1ra were infertile because of failure in the decidualization and, possibly, overinvasiveness of the trophoblast [36, 39]. Porcine embryos are capable of invading tissues when placed on ectopic locations [40]. Hence, noninvasiveness of the porcine implantation has been suggested to be controlled by the endometrium rather than by the trophoblast. The pregnancy-dependent localization of IL11RA to the surface epithelium suggests that IL11 signaling may play a role in the porcine endometrium by inhibiting the trophoblast invasion through the surface epithelium, similar to what is described in the mouse for the decidua zone.

It has been suggested previously that MUC4 expressed by the porcine endometrium also plays a role in protecting the surface epithelium from invasion [41]. Accordingly, in the present study, we identified MUC4 to have significantly higher expression in the pregnant compared to the nonpregnant endometrium. Experiments in rodents, on the contrary, show a down-regulation of mucins just before implantation of the blastocyst [42, 43]. The different placentation types between rodents and pigs, combined with the different expression pattern of MUC4, supports the idea that MUC4 might act as a protector of the porcine surface epithelium against invasion of the conceptuses [41].

On the contrary, the expression of MUC4 in the human endometrium is highest during the follicular (proliferative) phase of the menstrual cycle rather than during the luteal (secretory) phase [44]. Moreover, indications exist that MUC4 is not related to invasiveness in humans [45]. On the other hand, increased expression levels of MUC4 in cancers and hyperplastic diseases of the uterus instead support a role for MUC4 in regulating cell proliferation [44, 46].

The role of MUC4 as a regulator of differentiation and a modulator of proliferation can be explained by its C-terminal structure. It contains two epidermal growth factor (EGF)-like domains, which can interact with ERBB2 [47]. The receptor ERBB2 exhibits a strong kinase activity and is the preferred partner in forming dimers with other ERBB members [48]. The MUC4-ERBB2 complex interacts readily with ERBB3 in the presence of neuregulin, resulting in robust activation of the PI3K pathway and cell proliferation [49]. In the present study, we identified ERBB3 mRNA to have significantly higher expression in endometrium of pregnant compared to nonpregnant sows.

The mRNA coding for the growth factor FGF9 was significantly higher expressed in pregnant animals, and FGF9 has been identified previously as a growth factor in human endometrium [50]. The localization is, however, different. In pigs, the strongest staining for FGF9 was observed at the apical domain of the glandular epithelial cells; in humans, glandular epithelium only expresses the gene at low levels [50].

FGF9 binds with high affinity to FGFR2 and FGFR3 [51, 52]. In the human endometrium, FGF9 has no effect on proliferation of the epithelium despite the localization of FGFR2 in epithelial cells [50], indicating that FGF9 preferably acts through FGFR3 in the human endometrium. This may also be the case in the porcine endometrium, because another fibroblast growth factor, FGF7, has been identified as a likely candidate for FGFR2-mediated signaling [53]. Interestingly, the expression of FGFR3 mRNA was significantly lower in the pregnant sows, and FGFR3 staining was strongest in the glandular epithelium. This indicates the involvement of factors other than FGF9 in the regulation of proliferation of the surface epithelium. Moreover, FGF9 might act in an endocrine manner [54–56]. Hence, it could be speculated that an increase in secreted FGF9 from the endometrial glands during pregnancy may not be related to actions within the endometrium but, rather, may be implicated in the embryo-maternal communication.

In conclusion, we identified 263 genes to be differentially regulated in the endometrium of pregnant versus nonpregnant sows at the time of initial placentation. Furthermore, we showed a pregnancy-specific localization of IL11RA to the surface epithelium of the endometrium, suggesting that IL11 signaling may play an important role in formation of the porcine epitheliochorial placenta. We also showed that FGF9 is
up-regulated in pregnant endometrium, whereas its receptor, FGFR3, is down-regulated. Combined with the localization of FGF9 to the apical cell domain of the glandular epithelium, this finding makes it intriguing to speculate that FGF9 of endometrial origin functions as an embryonic growth factor in the pig.

ACKNOWLEDGMENT

The authors thank Danish Pig Production for arranging housing and insemination of the animals used in the present study.

REFERENCES

41. McNeer RR, Carraway CA, Fregien NL, Carraway KL. Characterization
48. Graus-Porta D, Beeri LL, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J 1997; 16:1647–1655.