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Differential Endometrial Gene Expression in Pregnant and Nonpregnant Sows[1]

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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 processes 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, FGFR3, 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 F_{2alpha} (PGF2a) [4]. Recent research indicates that the estrogen signal from the conceptuses stimulates endometrial prostaglandin E_2 (PGE2) synthesis. Combined with a positive PGE2 feedback loop in the endometrium, this synthesis leads to an increase in the PGE2:PGF2a ratio, which helps to overcome the luteolytic effect of PGF2a [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 accompanied 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-allantoic 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

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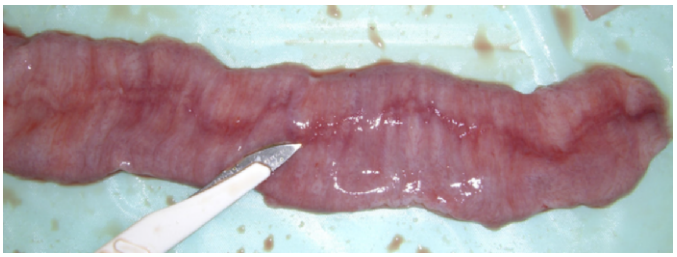

FIG. 1. Hyperemic zone in the endometrium identifying the sites of implantation, as seen after uterine flushing.

pregnancy-specific role(s) of estrogen- and interferon-stimulated genes in the porcine endometrium remains largely conjectural. Furthermore, only a few genes, which are 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 placentation.

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 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 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 involving 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.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18641). For quality control, normalized data were analyzed with a distance matrix and a heatmap based on pair-wise distances (BioConducter package geneplotter). Two samples were excluded from further analysis. Significance analysis was performed using the BioConductor package Linear Models for Microarray Data (LIMMA) [22]. Cut-off values were set to twofold difference in expression values and a false discovery rate of 1%. Identified probe sets were annotated by comparing two automated annotations [23, 24]. In case of disagreement between these annotations, the target sequences of the probe sets were manually annotated using BLAST [25]. If a gene was identified in the results by more than one probe set, the mean fold-change was calculated. Integrated analysis of different functional databases was done using the functional annotation clustering tool of the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [26].

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 ratio in the human genome is 127 (0.75%) of 16 968 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

TABLE 1. Primer sequences used to amplify specific fragments of porcine transcripts.

Gene symbol	PCR-product (bp)	Melting point (°C)	Primer sequence	Accession no.
UBB	85	81.6	Forward: 5′-GTCTGAGGGGTGGCTGCTAA-3′ Reverse: 5′-TGGGGCAAATGGCTAGAGTG-3′	NM_001105309
LIFR	152	77.1	Forward: 5′-GCAGGAAGCAAACTAGAGATTACAG-3′ Reverse: 5′-ACAGCAACATCACCTCCAAAT-3′	AJ656748
IL6R	107	80.0	Forward: 5′-GTTCCAATCCCTGCCTTCTG-3′ Reverse: 5′-CCCCAGTGCCAGTTTCTATG-3′	CF368332
IL11RA	95	83.9	Forward: 5′-GTGGATGGATGGACAGGACA-3′ Reverse: 5′-TGGCAGGTTTGGAACAGTC-3′	CK466326
FGF9	77	82.9	Forward: 5′-CTGGAAGGCGGTTCTATGTG-3′ Reverse: 5′-TTTCTGGTGCCGTTTAGTCC-3′	NM_213801
FGFR3	135	83.7	Forward: 5′-ATAGCCCGCACTACCTTTCA-3′ Reverse: 5′-GCCATGAGAACAGAGCCCTA-3′	CN163586
MUC4	84	81.8	Forward: 5′-AGCCATCACCCCTTCAACTC-3′ Reverse: 5′-TGGTAGGTTCTCACACAGACACA-3′	EV856946
ERBB3	140	78.6	Forward: 5′-CTCCCAACCTCTTTCTTCACA-3′ Reverse: 5′-TGACTTTGTTTCCCAGCAGA-3′	XM_001927652

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 carried out within 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 quantification (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) H_2O_2 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 placentation. Another group of enriched GO terms contains 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.

Primary antibody	Manufacturer	Product code	Source	Clonality	Isotype	Concentration (μg/ml)	HIER[a]	Primary antibody incubation[b]
Anti-human LIFR	R&D Systems (Minneapolis, MN)	AF-249-NA	Goat	Polyclonal	IgG	0.50	Ph 9	2 h RT
Anti-human IL6R	Santa Cruz (Santa Cruz, CA)	sc-661	Rabbit	Polyclonal	IgG	0.10	Ph 6	2 h RT
Anti-human IL11RA	Santa Cruz	sc-993	Rabbit	Polyclonal	IgG	8.00	Ph 9	2 h RT
Anti-human FGF9	Santa Cruz	sc-8413	Mouse	Monoclonal	IgG3	20.00	Ph 9	2 h RT
Anti-human FGFR3	Santa Cruz	sc-13121	Mouse	Monoclonal	IgG2a	8.00	Ph 9	2 h RT
Anti-human MUC4	Abnova (Taipei City, Taiwan)	H00004585-M07	Mouse	Monoclonal	IgG2a	0.20	Ph 6	2 h RT
Anti-human ERBB3	Santa Cruz	sc-285-G	Goat	Polyclonal	IgG	0.67	Ph 9	o/n 4°C

[a] HIER, heat induced epitope retrieval.
[b] RT, room temperature; o/n, over night.

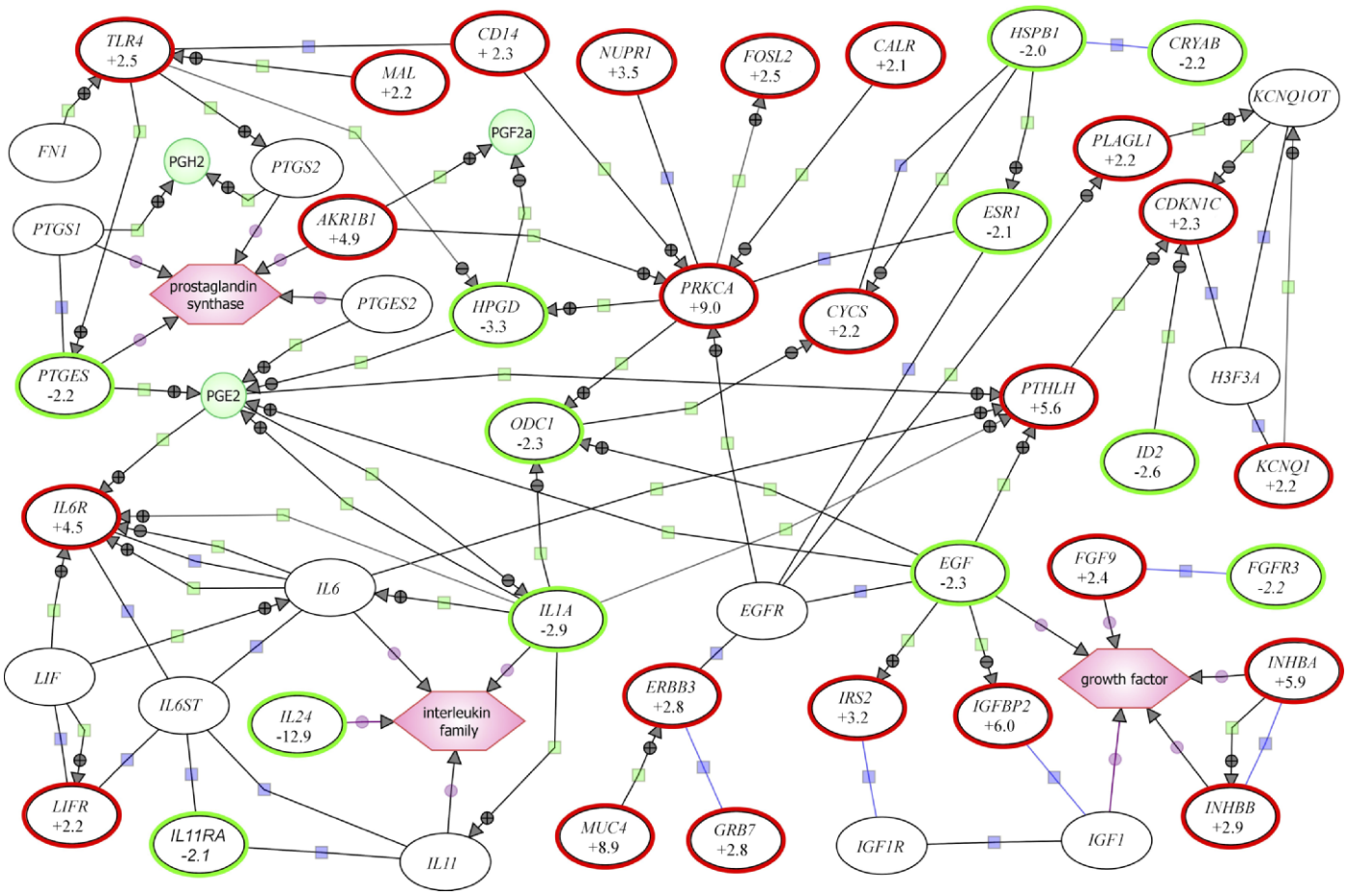

FIG. 2. Interaction network based on differentially expressed genes belonging to the GO term developmental process. Genes highlighted with red frames were up-regulated at pregnancy. Genes highlighted with green frames were down-regulated at pregnancy. The numbers under the gene symbols indicate the fold-change in mRNA expression. Genes framed with a thin black line were not identified to be differentially expressed in the endometrium when comparing pregnant and nonpregnant sows. Purple polygons illustrate gene families. Green circles illustrate hormones. Arrows with a green square indicate positive (+) or negative (–) regulation. Lines with a blue square indicate binding. Arrows with a purple circle indicate gene family member.

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).

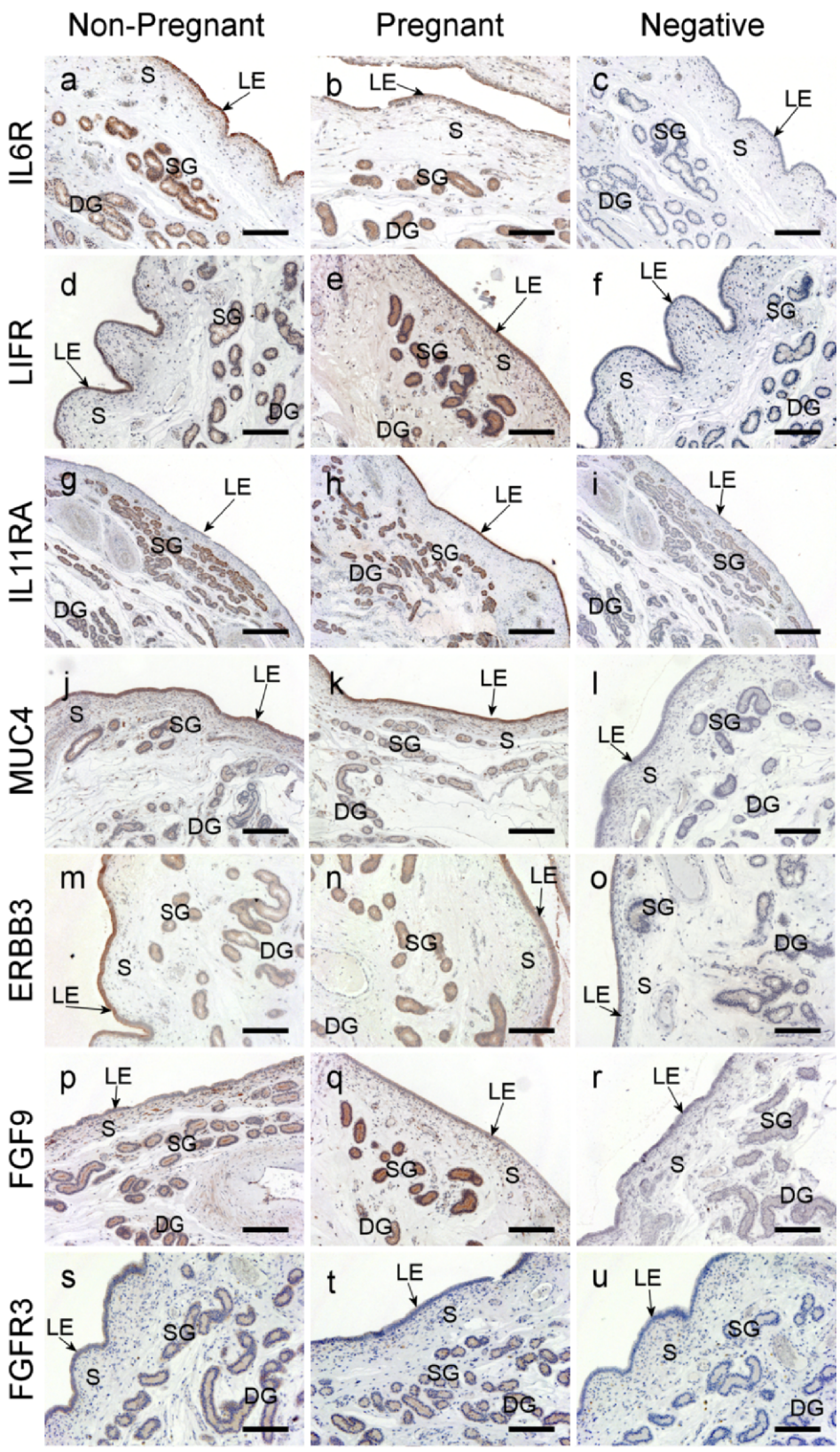

FIG. 3. Immunohistochemical localization of IL6R (**a** and **b**), LIFR (**d** and **e**), IL11RA (**g** and **h**), MUC4 (**j** and **k**), ERBB3 (**m** and **n**), FGF9 (**p** and **q**), FGFR3 (**s** and **t**), in endometrial tissue from nonpregnant (**a**, **d**, **g**, **j**, **m**, **p**, and **s**) and pregnant (**b**, **e**, **h**, **k**, **n**, **q**, and **t**) sows. Corresponding negative controls: IL6R preadsorption control (**c**), LIFR preadsorption control (**f**), IL11RA preadsorption control (**i**), murine IgG2a Isotype control (**l**), ERBB3 preadsorption control (**o**), murine IgG3 Isotype control (**r**), and murine IgG2a Isotype control (**u**). DG, deep glands; LE, luminal epithelium; S, stroma; SG, superficial glands. Bar = 200 μm (**a**–**f** and **j**–**u**) and 500 μm (**g**–**i**).

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.

TABLE 3. Significantly enriched gene ontology terms.

Gene ontology term	No. of genes	Genes[a]	Fold enrichment	*P* value for enrichment
GO:0032502 developmental process	66	***CD24, PRKCA, IGFBP2, PTHLH, INHBA, EDNRB, IL6R, EFHD1, LYZ, CLDN11, NUPR1, SCIN, IRS2, INHBB, ERBB3, LAMB3, SH3GL2, FOSL2, MTR, TLR4, HNF1B, ARSE, FGF9, FYN, ST6GAL1, PLXDC2, GJB3, RFFL, CDKN1C, GCLC, ENPP1, GULP1, CYCS, MAL, PLAGL1, CALR, IFI16, KLF9, FEZ2, BCL6, CD14, ITGAV, EDG1, SCML2, GNPTAB*** *RAB26, IL24, ANKH, TLL2, SDCBP2, ID4, HMGCR, IFI6, CHL1, IL1A, ID2, SLIT2, CRIM1, ODC1, EGF, RNF144B, CRYAB, FGFR3, CHRDL1, HSPB1, ESR1*	1.39	0.003
GO:0005215 transporter activity	31	***SLC16A1, TRPV6, CACNA1B, SLC2A1, STEAP1, SLC39A2, SLC11A2, FXYD3, MRS2L, ABCB10, SLC5A6, ATP10B, APOD, GJB3, NUP133, KCNQ1, MAL, AP1B1, CLIC2*** *FXYD4, AQP3, ANKH, ATP8A1, ATP1B1, SLC23A1, SLC9A4, SLC2A3, CCT6B, SLC7A9, STARD4, SLC25A1*	1.60	0.010
GO:0016491 oxidoreductase activity	27	***GPX3, KMO, AKR1B1, STEAP1, SOD3, COQ6, F5, IFI30, LTB4DH, CYCS*** *GRHPR, HPGD, SCD5, DHFR, HMGCR, SORD, SQLE, CYP2C18, HSD17B7, LDHD, SC4MOL, CYP2J2, CYP51A1, ETFB, RNF144B, FASN, MDH1*	2.15	0.000
GO:0005509 calcium ion binding	24	***S100A9, TRPV6, CAPNS1, PRKCA, CACNA1B, PTHLH, EFHD1, SCIN, F5, ARSE, KIAA0494, CALR, PROS1, MAN1A1, PLS3, ITGAV, GNPTAB*** *CALML4, TLL2, PADI2, PLCH1, ENTPD1, SLIT2, EGF*	1.81	0.007
GO:0006629 lipid metabolic process	23	***PLCXD3, APOD, LTB4DH, PLA2G7*** *FDPS, HPGD, SCD5, HMGCR, PIGB, SQLE, PLCH1, HSD17B7, HMGCS1, IDI1, ACAT2, SC4MOL, CYP2J2, CYP51A1, STARD4, MTMR7, LSS, PTGES, FASN*	2.06	0.002
GO:0006915 apoptosis	22	***CD24, PRKCA, INHBA, NUPR1, SCIN, RFFL, GCLC, GULP1, CYCS, MAL, PLAGL1, CALR, IFI16, BCL6, CD14*** *IL24, IFI6, IL1A, RNF144B, CRYAB, HSPB1, ESR1*	1.92	0.005
GO:0009605 response to external stimulus	22	***S100A9, NPY, STC1, PRKCA, INHBA, LYZ, INHBB, F5, TLR4, ENPP1, PROS1, BCL6, CD14, PLA2G7, LY96, EDG1, VWF*** *DEFB1, IL1A, ENTPD1, SLIT2, GPR68*	2.34	<0.001
GO:0006082 organic acid metabolic process	21	***PRKCA, MTR, GCLC, LTB4DH, ASS1, CAD*** *HDC, BCAT1, GRHPR, GOT1, HPGD, SCD5, DHFR, SLC7A9, SC4MOL, CYP2J2, EGF, PTGES, ACO1, FASN, MDH1*	2.50	<0.001
GO:0006928 cell motility	14	***CD24, SELL, NPY, PRKCA, EDNRB, FYN, GAB1, FEZ2, BCL6, EDG1*** *HMGCR, CHL1, SLIT2, HSPB1*	2.28	0.009
GO:0007167 enzyme linked receptor protein signaling pathway	14	***PRKCA, INHBA, IRS2, ERBB3, GRB7, FGF9, CDKN1C, GAB1*** *SORBS1, FMOD, HPGD, EGF, CRYAB, FGFR3*	3.37	0.000
GO:0008284 positive regulation of cell proliferation	11	***CD24, CAPNS1, PTHLH, IRS2, FGF9, LIFR, BCL6, EDG1*** *ID4, ODC1, EGF*	3.14	0.003
GO:0050801 ion homeostasis	11	***CD24, STC1, PRKCA, EDNRB, AGTR1, TFRC, GCLC, CALR, EDG1*** *SLC9A4, IFI6*	3.20	0.002
GO:0005179 hormone activity	8	***NPY, NMB, STC1, PTHLH, INHBA, INHBB*** *PENK, CORT*	4.69	0.002

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

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

	Relative expression (mean ± SEM)		Mean fold changes		*P* value	
Genes	Pregnant	Nonpregnant	qPCR	Microarray	qPCR	Microarray
MUC4	1.66 (0.30)	0.08 (0.02)	19.7	8.9	0.004	<0.001
ERBB3	2.09 (0.45)	0.55 (0.10)	3.8	2.8	0.004	0.001
LIFR	1.53 (0.17)	0.59 (0.04)	2.6	2.2	0.004	0.005
IL6R	2.14 (0.25)	0.38 (0.08)	5.7	4.5	0.004	0.006
IL11RA	2.15 (0.51)	4.90 (0.40)	−2.3	−2.1	0.008	0.006
FGF9	1.86 (0.26)	0.45 (0.03)	4.1	2.4	0.004	0.001
FGFR3	0.70 (0.08)	1.03 (0.10)	−1.5	−2.2	0.048	0.003

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 placentation.

The interleukin 6 family of cytokines is well known to be a key regulator of implantation and placentation. 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 the LIFR 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 *Il11ra* knock-out mice. Hence, mice lacking *Il11ra* 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 decidual 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 fibroblastic 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.

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