Modulation of peroxisome proliferator-activated receptor δ and γ transcripts in swine endometrial tissue during early gestation

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Abstract

Recent evidence points to a role for peroxisome proliferator-activated receptors (PPARs) δ and γ in embryo implantation and survival. In this study, we report the porcine PPARδ complete coding sequence and mRNA abundance of PPARδ, PPARγ1 and γ2, angiopoietin-like protein 4 (ANGPTL4) and adipocyte determination and differentiation-dependent factor 1 (ADD1) genes in the pregnant sow endometrium. Real-time PCR analysis was used to study the effect of parity (Yorkshire-Landrace multiparous (YL) and nulliparous (YLn)), site of endometrial tissue sampling (between and at embryo attachment sites) in crossbred Duroc×Yorkshire-Landrace (DYL) sows and stages of pregnancy (non-pregnant, day 15 and day 25 after mating) in Meishan-Landrace (ML) on mRNA levels. Parity effects were observed for PPARδ, ANGPTL4, and ADD1, with higher mRNA levels in YL than YLn sows. In DYL sows, lower mRNA levels were present at attachment sites compared to between attachment sites for PPARδ, PPARγ1, and ANGPTL4. Finally, day 15 pregnant ML sows had lower PPARδ mRNA levels compared to day 15 cycling ML sows. A significant increase of PPARγ1 mRNA levels was found on day 25 pregnant ML and DYL sows relative to day 15 ML or DYL pregnant sows. PPARδ and γ immunostaining was detected in endometrial tissue of day 15 cycling sows, day 15 and 25 pregnant sows and epithelial cells of day 25 embryos. Collectively, our results suggest a role for PPARδ, PPARγ1, and ANGPTL4, but not PPARγ2, during the peri-implantation period in pregnant sows.


Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-dependent transcription factors that regulate the expression of target genes involved in cell differentiation and proliferation (Rosen et al. 2000, Michalik et al. 2002). Three subtypes of PPARs, named PPARα, PPARδ/β, and PPARγ (PPARγ1 and γ2), have been identified in vertebrates, each encoded by a separate gene and fulfilling distinct functions. Whereas the importance of PPARs in the control of lipid and glucose homeostasis and cellular growth and differentiation has been well established, much less is known about their function in reproductive tissues. PPARδ is highly expressed in the mouse uterus at implantation sites and its expression requires the presence of an active blastocyst (Lim et al. 1999, Ding et al. 2003b). PPARδ was also found in the rat uterus at implantation sites and in decidual cells (Ding et al. 2003a). PPARγ is expressed in human (Marvin et al. 2000), rat (Asami-Miyagishi et al. 2004), and mouse (Barak et al. 1999) placental tissues at various stages of gestation. Studies from knockout mouse models showed that both PPARδ and PPARγ have essential, but different roles in murine pregnancy. Indeed, while PPARγ appears to be required for the differentiation of the placenta, PPARδ seems to be more important for normal development of the placental-decidual interface (Barak et al. 1999, 2002). Normal fertility was reported in PPARδ deficient mice, thus suggesting that this nuclear receptor is not essential for normal reproductive function (Lee et al. 1995).

The observation that uterine cyclo-oxygenase-2 (COX-2), a rate limiting enzyme in prostaglandin synthesis, is expressed in an implantation-specific
manner and that implantation is defective in COX-2-deficient mice established that uterine prostaglandins produced by COX-2 may play an essential role in this uterine event (Lim et al. 1997). Moreover, the PPARδ selective agonist, L-165041, is able to restore implantation in COX-2-deficient mice, suggesting that COX-2-derived prostaglandins mediate embryo implantation via PPARδ (Lim et al. 1999). Numerous arachidonic acid metabolites have been shown to be naturally occurring ligands for nuclear PPARs (reviewed in Helliwell et al. 2004). Prostaglandins are essential during early porcine pregnancy for vascular permeability, placenta development, and immune responses (Geisert et al. 1990), suggesting a key role for PPARγ and δ in early gestation in pigs. Various genes contain peroxisome proliferator response elements (PPREs) in their upstream regulatory sequences (Martin et al. 1997); however, biologically relevant target genes are largely unknown in reproductive tissues. Angiopoietin-like protein 4 (ANGPTL4) is a PPARγ target gene (Mandard et al. 2004) and, due to its previously reported role as an apoptosis survival factor (Kim et al. 2000) and in angiogenesis (Belanger et al. 2002, Le Jan et al. 2003), it is a relevant PPAR target gene to study in early pregnancy. Therefore, the objectives of this study were (1) to evaluate whether PPARδ, PPARγ1, PPARγ2, and ANGPTL4 are expressed in pig endometrium; (2) to examine the effects of the day of pregnancy (non-pregnant, day 15 and day 25), parity of the sows, and site of endometrial tissue sampling on PPARδ, PPARγ1, PPARγ2, and ANGPTL4 mRNA levels in endometrial tissues. In addition, mRNA levels of ADD1/SREBP1c, a member of the basic helix–loop–helix transcription factor family, were also estimated in this experimental design. This last candidate was chosen because of its previously reported role in the production of endogenous ligands for PPARγ (Kim et al. 1998b).

Materials and Methods

Animals

Three groups of sows (Table 1) were kindly provided by Genetiporc (St-Bernard, QC, Canada). The first group comprised 22 multiparous Yorkshire-Landrace sows (YL, 4–5 parities) and 22 nulliparous Yorkshire-Landrace sows (YLn) and was used to study parity effects on transcript abundance of selected genes. The second group consisted of 24 crossbred Duroc×Yorkshire-Landrace sows (two parities) that were killed on day 15 (DYL15, n=14) or on day 25 (DYL25, n=10) of gestation. These sows were used to study the effects of day of pregnancy and site of endometrial tissue sampling (at implantation or between implantation sites). These sows were also used for immunohistochemical analysis. A third group of 24 Meishan-Landrace sows were killed on day 15 (ML15, 2–3 parities) or on day 25 (ML25, 2–3 parities) of gestation, and six Meishan-Landrace sows were killed on day 15 of the estrous cycle (MLc, 2–3 parities). This last group was used to study the effects of pregnancy status (day 15 pregnant vs day 15 of the estrous cycle) and of the day of pregnancy (day 15 vs day 25 pregnant sows). A crossbred sow was also used for tissue distribution analysis. Heat detection was performed twice a day, between 0800 and 0900 h and between 1600 and 1700 h, by introducing a boar into the pen. At the second estrus, sows were inseminated twice with pooled semen from Landrace boars of proven fertility (CIPQ, Inc., St-Lambert, QC, Canada), 12 and 24 h after estrus detection. The day of estrus was considered day 0. All procedures involving animals were approved by the local Animal Care Committee following the guidelines of the Canadian Council on Animal Care (1993).

Tissue collection

Prior to collection of endometrial tissue, the uteri of day 15 pregnant sows were flushed with PBS and pregnancy confirmed by the presence of embryos in the flushing. The reproductive tract was collected and the uterine horns were opened along the antimesometrial border. Endometrial tissue samples were taken from the mesometrial side, at the sites or between sites of conceptus attachment for the pregnant sows, or in the middle of the horn for the cyclic sows. A minimum of five attachment sites or five endometrial sites were collected for each sow. Attachment sites from day 15 sows were determined by the presence of local hyperemia in endometrial tissue, manifest as a darker reddish color compared to surrounding tissue. All tissue samples were immediately frozen in liquid nitrogen and stored at

<table>
<thead>
<tr>
<th>Groups</th>
<th>Breed and parity of sows</th>
<th>Days of pregnancy (P) or estrous (E) cycle</th>
<th>Sows (n)</th>
</tr>
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<tr>
<td>1</td>
<td>Yorkshire-Landrace multiparous (YL)</td>
<td>P d25</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Yorkshire-Landrace nulliparous (YLn)</td>
<td>P d25</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>Duroc×Yorkshire-Landrace multiparous (DYL15)</td>
<td>P d15</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Duroc×Yorkshire-Landrace multiparous (DYL25)</td>
<td>P d25</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Meishan-Landrace multiparous (MLc)</td>
<td>E d15</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Meishan-Landrace multiparous (ML15)</td>
<td>P d15</td>
<td>13</td>
</tr>
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<td></td>
<td>Meishan-Landrace multiparous (ML25)</td>
<td>P d25</td>
<td>11</td>
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</table>
−80 °C until processed. The procedure was completed within 20 min after slaughter. Several tissues were sampled, including liver, kidney, heart, lung, ovary, stomach, brain cortex, endometrium, skeletal muscle, ham subcutaneous fat, back subcutaneous fat, and visceral fat.

**RNA extraction and complementary DNA preparation**

Total RNA was extracted from endometrial tissue (minimum of five samples per sow) using TRIzol Reagent (Gibco BRL) according to the manufacturer's instructions. Endometrial RNA samples from individual animals were pooled prior to cDNA synthesis. Total RNA was dissolved in water and quantified spectrophotometrically at 260 nm and an RNA aliquot was run on 1% agarose gel to verify its integrity. Total RNA was reverse transcribed to cDNA in a PTC-200 Peltier Programmable Thermal Cycler (MJ Research, Foster City, CA, USA). Five micrograms of total RNA were treated with three units of Dnase I (amplification grade; Gibco BRL) to remove contaminating genomic DNA. First-strand cDNA was synthesised using a SuperScript II preamplification system (Gibco BRL) and 500 ng of oligo(dT) 12–18 as primer (Amersham Pharmacia Biotech) in a 50 µl reaction volume.

**Cloning and sequencing of porcine PPARδ**

To determine the porcine specific sequence of PPARδ, degenerate primers (PPARD-F and PPARD-R; Table 2) were designed based on homology between human (GenBank accession no. HUMPPARA), rat (GenBank accession no. U75918), and mouse (GenBank accession no. NM_011145) sequences. PCR amplification was performed under the following conditions: the 50 µl PCR reaction contained 200 µM dNTPs, 300 nM of each primer, 1.5 mM MgCl2, 1 unit of Taq polymerase (Clontech) in 1× Taq polymerase buffer. The PCR profile consisted of an initial denaturation step at 94°C for 1 min followed by 33 cycles of 94°C for 30 s, 55°C for 30 s, 68°C for 2 min, and a final extension at 68°C for 3 min. This amplification generated one fragment of 576 bp. The nucleotide sequence of the amplified fragment was determined by cycle sequencing in both directions – a total of three independent amplifications. Sequence determination was performed using the Big Dye Terminator Cycle Sequencing Ready Reactions (PE Applied Biosystems, Foster City, CA, USA) according to manufacturer’s instructions and run on an ABI 377 DNA sequencer (PE Applied Biosystems).

To obtain the complete coding sequence of porcine PPARδ, 5’ and 3’ rapid amplification of the cDNA ends (RACE) was performed using the Marathon cDNA amplification kit (Clontech). Poly A+ RNA was isolated using the Nucleotrap mRNA purification kit (Clontech). One microgram of Poly A+ RNA from sow endometrial tissue was reverse-transcribed using the Marathon cDNA synthesis primer. Second strand cDNA was synthesized and a Marathon cDNA adaptor (Clontech) was ligated as recommended by the manufacturer. The 3’ end amplification of porcine PPARδ was performed using an upstream primer 5’-ACTACGGAGTCCACGCTTGCGA-3’ which corresponds to pig PPARδ cDNA between nucleotides 535 and 556 (GenBank accession no. NM_214152). The 3’ RACE generated a single fragment of 1.5 kb.

**Table 2 Oligonucleotide primers used for cloning, tissue distribution, and real-time PCR of selected porcine genes.**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences (5’–3’)a</th>
<th>Primer position (nt)</th>
<th>GenBank accession no.</th>
<th>Length (bp)</th>
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<tbody>
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<tr>
<td>PPARD-F</td>
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<td>CTGAAGYTGGGATTCTGCTTGG</td>
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<td>Tissue distribution</td>
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<td>PPARD-t-F</td>
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<tr>
<td>ADD1-t-F</td>
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<tr>
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<td>ANGPTL4-t-F</td>
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<tr>
<td>PPARD-rt-F</td>
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<td>71</td>
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<tr>
<td>PPARD-rt-R</td>
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<td>Cyclophilin-F</td>
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<td>AY266299</td>
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<td>Cyclophilin-R</td>
<td>AGGCACGGATGCGTTCAAGA</td>
<td>270–289</td>
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F, forward; R, reverse.

*aDegenerated primers where M=A or C, R=A or G, Y=C or T.
The 5' end of pig PPARδ was amplified using a pig specific downstream primer 5'-CTGCCAACAG- TCTGGATGTCG-3', which hybridizes with nucleotides 923 to 944 of the pig PPARδ cDNA sequence (GenBank accession no. NM_214152). The amplified 5' end generated a single fragment of 1 kb. Both 5' and 3' end fragments were sequenced as described above and were assembled using the AutoAssembler 2.0 software (PE Applied Biosystems) to determine the complete porcine specific PPARδ coding sequence. PPARδ sequence features were found using NCBI Conserved Domain Search (Marchler-Bauer et al. 2003).

Tissue distribution of porcine PPARδ, ANGPTL4, and ADD1 mRNA

Amplifications of PPARδ, ADD1, and ANGPTL4 cDNAs were performed in various pig tissues using primers described in Table 2. Total RNA extraction and cDNA synthesis were performed as described above. PCR amplifications were performed in a 100 µl total volume which contained a 2 µl aliquot of the reverse transcriptase product, 150 nM of forward and reverse primers, 200 µM dNTPs, 1 mM MgCl₂ (0.8 mM for ADD1), and 0.5 unit of Taq polymerase in 1×Taq polymerase buffer (Amersham Pharmacia Biotech). The PCR profile consisted of an initial denaturation step at 94 °C for 2 min, followed by 35 cycles (36 for ADD1) of denaturing at 94 °C for 1 min, annealing at 69 °C (61 °C for ADD1 and ANGPTL4) for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. Pig cyclophilin amplification using forward 5'-ACCGTCTTTAAGA- CATCGC-3' and reverse 5'-CTTGCTGTTTTGCCA- TTCC-3' primers, which correspond to nucleotides 17–36 and 447–466 of the pig cyclophilin sequence (GenBank accession no. AY266299), was also performed on the same cDNAs as an internal control for variations in cDNA synthesis. The 100 µl PCR reaction mixture contained a 2 µl aliquot of the reverse transcriptase product, 150 nM of forward and reverse primers, 200 µM dNTPs, 1.0 mM MgCl₂, and 0.5 unit of Taq polymerase in 1×Taq polymerase buffer (Amersham Pharmacia Biotech). The PCR profile used was the same as that described above for PPARδ, with the exception of the annealing temperature, which was 60 °C. Amplified PCR fragments were electrophoresed on a 1.5% agarose gel (3% for ADD1) and stained with ethidium bromide. These fragments were single pass sequenced as described above to confirm their identity. Pictures of the resulting gels were taken with Polaroid positive/negative film (#55). Negatives from Polaroid positive/negative photos were then scanned using an Imaging Densitometer (Model GS-670; BioRad). PCR amplifications were repeated in duplicate using two different tissue samples from the same animal.

Quantitative measurements of mRNA levels in endometrial tissues

Endometrial tissues at embryo attachment (groups 1, 2, and 3) and between attachment sites (group 2) were analyzed for PPARδ, PPARγ1, PPARγ2, ADD1, and ANGPTL4 mRNA levels using real-time PCR amplifications. Gene-specific primers (Table 2) were designed and selected using the Primer Express Software (PE Applied BioSystem). Primers used for ADD1 are the same as those used for tissue distribution. Real-time PCR amplifications were performed in 25 µl reaction volume consisting of 50 nM of forward and reverse primer, 1 µl of cDNA, 0.25 µl AmpErase (PE Applied BioSystems) and 1×SYBR Green Master Mix (PE Applied BioSystems). Cycling conditions were 2 min at 50 °C, followed by 10 min at 95 °C. Then, 40 cycles of 15 s at 95 °C and 1 min at 60 °C were performed. Amplification, detection, and analysis were performed with an ABI Prism 7700 Sequence Detector (PE Applied BioSystems). Samples were normalised using the housekeeping gene cyclophilin (Table 2). Reaction mixtures contained 50 nM of forward and 900 nM of reverse primers. Real-time PCR cycling conditions were the same as described above. All real-time PCR reactions were performed in triplicate and standard curves were established in duplicate for each gene. Target genes and cyclophilin amplifications were run in separate assays. A pool of endometrial cDNA was used to create a standard curve for quantification of the transcripts using the relative standard curve method as described by Applied Biosystems (User Bulletin #2 1997). Standard curve arbitrary units were set at 1 for the undiluted cDNA pool and dilutions of 0.75, 0.50, 0.25, 0.10, 0.05, 0.025, and 0.005 were then performed. For each experimental sample, the amount of target gene mRNA relative to endogenous cyclophilin was determined from their respective standard curves. Relative quantity ratios were obtained by dividing the relative quantity units of target genes by those of cyclophilin. Mean values from triplicates were then used to perform statistical analyses. The specificity of the amplified fragments was verified on a 3.5% agarose gel and with melting curve analysis using the Dissociation Curves v1.0 software (PE Applied BioSystems).

Immunohistochemical analysis

Uterine tissues were fixed in 4% paraformaldehyde, 0.1 M sodium cacodylate and kept in a 0.4% paraformaldehyde solution at 4 °C until embedded in paraffin. Endogenous peroxidase activity was quenched by incubating slides for 30 min in methanol containing 0.3% hydrogen peroxide, and sections were boiled for 15 min in 0.01 M citrate buffer, pH 6.0 for antigen retrieval. Non-specific protein binding was blocked by incubating sections in Tris-buffered saline (TBS) containing 10% normal goat serum (Jackson ImmunoResearch...
Laboratories, West Grove, PA, USA) for 45 min at room temperature. Tissues were then incubated overnight at 4°C in a 1:50 dilution in TBS of either rabbit anti-human PPARα or PPARγ polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Slides were washed twice (5 min) at room temperature. Primary antibody was detected using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA), which includes a biotinylated goat anti-rabbit secondary antibody at a concentration of 10 μg/ml. The Nova Red substrate kit (Vector Laboratories) detected peroxidase activity in the form of a red precipitate. Sections were counterstained with hematoxylin. Control sections were subjected to the same procedure, except that rabbit anti-human PPARγ and PPARα antibodies were omitted.

**Statistical analyses**

Relative quantification of mRNA levels was performed according to the standard curve method described by Applied Biosystems (User Bulletin no. 2, PE Applied Biosystem 1997). Group 1 means were analyzed using a one-way ANOVA on two treatment groups (multiparous vs nulliparous). Groups 2 and 3 means were analyzed by all pair-wise multiple comparison procedures (Tukey test) or by a two-way ANOVA (full factorial on sampling site and day of pregnancy) where pertinent. Data were analyzed using SAS (SAS Institute, Inc., Cary, NC, USA, version 8.1) and results are presented as least square means of mRNA relative abundance ± S.E.M. Statistical significance was set at P < 0.05.

**Results**

**Cloning and sequence analysis of porcine PPARδ cDNA**

Using RT-PCR and RACE on total RNA isolated from porcine endometrial tissue, the complete cDNA coding sequence for porcine PPARδ was obtained (GenBank accession no. NM_214152). Analysis of the porcine PPARδ nucleotide sequence indicated it to be 91.7, 88.1, and 88.8% identical to the human, rat, and mouse sequences, respectively, with a predicted protein sequence length of 441 amino acids. Analysis of the deduced PPARδ amino acid sequence (Fig. 1) showed a 95, 89, and 90% homology with human, rat, and mouse sequences respectively. Multiple alignments revealed that the DNA-binding and ligand-binding domains, characteristic of the nuclear receptors, are also well conserved in the pig PPARδ protein relative to other species (Desvergne & Wahli 1999).

![Figure 1](https://www.reproduction-online.org)
Expression of PPARδ, ANGPTL4, and ADD1 transcripts in porcine tissues

Relative abundance of PPARδ, ANGPTL4, and ADD1 mRNA were assessed in various tissues from an adult sow. A single amplified fragment of 358 bp was detected for PPARδ in all tissues tested (Fig. 2). ANGPTL4 amplification generated a single fragment of 340 bp, which was expressed in all tissues with the exception of the cerebral cortex where it was undetectable. Finally, a single ADD1 fragment of 79 bp was amplified in all tissues but very faint amplifications were obtained in the heart and brain cortex tissues.

PPARδ and PPARγ localization during the peri-implantation period

Immunostaining detected PPARδ and PPARγ in nuclei of pig endometrial cells while both were absent in the myometrium. On day 15 of pregnancy, PPARδ staining was observed at attachment sites in the luminal and glandular epithelium and minor expression of PPARδ was present in the subepithelial stroma (Fig. 3A and B). Between attachment sites, there was PPARδ immunoreactivity in the luminal and glandular epithelium, but none in stromal cells (Fig. 3C and D). On day 25 of pregnancy, PPARδ protein was found in trophoblast cells (Fig. 3E) but, at the implantation sites, only weak expression was detected in the luminal epithelium and it was absent from the glandular epithelium and stroma (Fig. 3E and F). Between attachment sites, a stronger signal was detected for PPARδ in the luminal epithelium and staining was detected in the glands located in close proximity to the luminal epithelium (Fig. 3G). No signal could be detected in glands adjacent to the myometrium (Fig. 3H). Day 15 cycling sows displayed PPARδ immunostaining in luminal and glandular epithelium but we were unable to detect a signal in stromal cells (Fig. 3I and J).

The antibody employed interacted with both isoforms of PPARγ, and thus it was not possible to distinguish between these subtypes. Readily identifiable immunostaining was present in endometrial luminal and glandular epithelium at day 15 in cycling sows (Fig. 4I and J) and pregnant sows both between (Fig. 4C and D) and in attachment sites (Fig. 4A and B). Faint PPARγ staining was detected in subepithelial stroma at the implantation sites only (Fig. 4A). On day 25 of pregnancy, PPARγ protein was detected in trophoblast cells (Fig. 4E), in cells of the luminal epithelium at (Fig. 4E) and between (Fig. 4G) attachment sites. The glandular epithelium of day 25 pregnant sows showed no PPARγ immunostaining at attachment sites (Fig. 4F) and only weak signal between attachment sites (Fig. 4H). No staining was evident in stromal cells at this time (Fig. 4E–H).

Effect of parity on mRNA abundance of selected genes in endometrial tissues

Using real-time PCR, porcine PPARδ, PPARγ1, PPARγ2, ANGPTL4, and ADD1 mRNA levels were quantified in endometrial tissue collected at attachment sites from sow group 1. Levels of PPARδ mRNA were higher in multiparous than in YLn sows (Fig. 5A; \( P < 0.01 \)). Significant parity effects were also observed for ANGPTL4 (Fig. 5D; \( P < 0.01 \)) and ADD1 (Fig. 5E; \( P < 0.05 \)) mRNA levels, where they were found to be higher in YL compared with the nulliparous sows. There were no significant parity effects for porcine PPARγ1 and PPARγ2 mRNA levels (Fig. 5B and C; \( P > 0.05 \)).

Effect of the site of endometrial tissue sampling

To determine whether porcine PPARδ, PPARγ1, PPARγ2, ANGPTL4, and ADD1 mRNA levels could be modulated according to the site of endometrial tissue sampling, tissues were collected at and between embryo attachment sites. Expression levels of PPARδ, PPARγ1, and ANGPTL4 mRNAs were higher between sites relative to attachment sites and this effect was significant at both day 15 and day 25 of pregnancy (Fig. 6A, B, and D; \( P < 0.05 \)). In contrast, there was no effect of endometrial tissue site sampling on PPARγ2 and ADD1 mRNA levels at either day 15 or day 25 of pregnancy (Fig. 6C and E; \( P > 0.05 \)).

Effect of the day of pregnancy and pregnancy status on selected gene mRNA levels

The effect of the day of pregnancy and pregnancy status on expression levels of PPARδ, PPARγ1, PPARγ2, ANGPTL4, and PPARγ1, ANGPTL4, and ADD1 mRNAs in various tissues collected from an adult Duroc X Yorkshire-Landrace sow. Li, liver; Ki, kidney; He, heart; Lu, lung; Ov, ovary; St, stomach; Br, brain cortex; En, endometrium; Sk, skeletal muscle; Hf, ham subcutaneous fat; Bf, back subcutaneous fat; Vf, visceral fat; Ne, negative control without cDNA; Ma, molecular marker. Expected fragment length (bp) is indicated on the right. Equal amounts of PCR products were loaded per lane as adjusted by cyclophilin housekeeping gene.

Figure 2 RT-PCR analysis of PPARδ, ANGPTL4, and ADD1 mRNA in various tissues collected from an adult Duroc X Yorkshire-Landrace sow. Li, liver; Ki, kidney; He, heart; Lu, lung; Ov, ovary; St, stomach; Br, brain cortex; En, endometrium; Sk, skeletal muscle; Hf, ham subcutaneous fat; Bf, back subcutaneous fat; Vf, visceral fat; Ne, negative control without cDNA; Ma, molecular marker. Expected fragment length (bp) is indicated on the right. Equal amounts of PCR products were loaded per lane as adjusted by cyclophilin housekeeping gene.
and ADD1 mRNAs was studied in Duroc×Yorkshire-Landrace (group 2) and in Meishan-Landrace (group 3) sows. In the Meishan-Landrace group, the expression level of PPARδ mRNA was higher in day 15 cycling sows compared with day 15 (P<0.05) pregnant sows (Fig. 7A). In both groups 2 and 3, there were no significant differences in PPARδ mRNA levels between day 15 and day 25 pregnant sows (Figs 6A and 7A; P>0.05). PPARγ1 mRNA levels were higher at day 25 than at day 15 of pregnancy for both group 2 (Fig. 6B, P<0.05) and group 3 (Fig. 7B, P<0.01) sows, but there was no significant difference of PPARγ1 mRNA levels between day 15 cycling sows and day 15 pregnant sows (Fig. 7B; P>0.05). There were no effects of the day of

Figure 3 Immunohistochemical localization of PPARδ in pig uterus. (A) and (B) Attachment sites of a day 15 pregnant sow; PPARδ protein is localized in luminal and glandular epithelium; minor staining is detected in the first cell layer of subepithelial stroma. (C) and (D) Tissue from between attachment sites of a day 15 pregnant sow; PPARδ is detected in luminal and glandular epithelium. (E) and (F) Attachment sites of a day 25 pregnant sow; PPARδ immunostaining present in trophoblast and weak signal is detected in the luminal epithelium; PPARδ protein is absent from stroma and glands. (G) and (H) Between attachment sites of a day 25 pregnant sow; PPARδ is observed in the luminal epithelium and in glands located near the luminal epithelium; no signal detected in glands adjacent to the myometrium. (I) and (J) Day 15 cycling sow; immunostaining is present in luminal and glandular epithelium. (K) and (L) Negative controls for localization. e, luminal epithelium; g, glandular epithelium; s, stroma; my, myometrium; mv, maternal blood vessel; t, trophoblast. Bars = 80 μm.
pregnancy or pregnancy status on PPARγ2, ANGPTL4, and ADD1 mRNA levels in endometrial tissue for both groups 2 and 3 (Fig. 6C–E and Fig. 7C–E; P > 0.05).

**Discussion**

To our knowledge, this is the first report of the cloning and characterization of pig PPARδ and the first to study mRNA and protein expression of PPARs in pig endometrial tissues. The complete coding sequence of the porcine PPARδ was generated by RT-PCR and RACE using RNA from endometrial tissue as the template. A multiple sequence alignment of porcine PPARδ protein with its human, rat, and mouse ortholog shows that the 1–71 amino acids N-terminal extension is the least conserved region with 10 non-conserved amino acids.
relative to the human PPARδ protein sequence. In contrast, the DNA-binding domain is the most conserved region with only one non-conserved amino acid relative to the human PPARδ protein sequence. This domain is also known to be the most conserved domain among the nuclear receptor superfamily (Desvergne & Wahli 1999). The porcine PPARδ ligand-binding domain shows a seven amino-acid difference from its human counterpart. This species discrepancy may have functional relevance to the ability of porcine PPARδ to bind ligands differentially, as it was previously demonstrated that PPARα isotypes from Xenopus, mouse, and human respond differentially to the PPARα ligands Wy14,643 and ETYA (Keller et al. 1997).

The wide tissue distribution of porcine PPARδ transcript contrasts with the more restricted expression of porcine PPARα (Sundvold et al. 2001) and PPARγ mRNA (Grindflek et al. 1998). The ubiquitous expression of PPARδ mRNA in porcine tissues is in accordance with previous studies in human (Skogsberg et al. 2000), rat (Kliewer et al. 1994, Xing et al. 1995), and mouse (Amri et al. 1995). ADD1 and ANGPTL4 transcripts were detected in pig endometrium and ovaries for the first time, suggesting a putative role for these genes in reproductive tissues. Previous studies have reported that the ANGPTL4 transcript was highly enriched in mouse white and brown adipose tissues and in placenta, whereas a lesser mRNA expression was observed for

Figure 5 Parity effects on relative expression of PPARδ (A), PPARγ1 (B), PPARγ2 (C), ANGPTL4 (D), and ADD1 (E) mRNA in endometrial tissue from day 25 pregnant Yorkshire-Landrace sows. Data are ratios of selected genes relative mRNA levels normalized to cyclophilin housekeeping gene mRNA levels. Each bar represents least square means ± S.E.M. *P<0.05; **P<0.01.
liver, kidney, heart, and lung (Kersten et al. 2000, Yoon et al. 2000). Interestingly, ANGPTL4 mRNA tends to be more widely and evenly expressed in pig tissues, with the exception of the brain cortex for which no transcript could be detected. As previously reported for human, mouse, and rat tissue distribution studies (Tontonoz et al. 1993, Shimomura et al. 1997, Kim et al. 1998a), we were able to detect ADD1 transcript in pig liver, kidney, lung, ovary, skeletal muscle, adipose tissues, heart, and brain. Our results are in contrast with those of Ding et al. (1999, 2000), who reported ADD1 transcripts to be undetectable in pig kidney and skeletal muscle. In the current study, we used an adult sow to perform tissue distribution analysis, whereas Ding et al. (1999, 2000) used much younger pigs (20–30 kg). Thus, it would be of interest to further study pig ADD1 transcripts in these tissues through musculoskeletal maturation.

PPARs were first recognized as key regulators of adipose differentiation and glucose homeostasis (Desvergne & Wahli 1999), but accumulating evidence points towards a functional role of the PPARs in the female reproductive tract. For instance, PPARγ-null mice exhibit placental defects in trophoblast differentiation and vascular processes (Barak et al. 1999). Further, 90% of the PPARγ (−/−) mouse embryos have severe developmental defects, principally due to placental malformation (Barak et al. 2002). Although, the role of PPARs in fatty acid metabolism has been demonstrated

**Figure 6** Effects of the day of pregnancy and site of endometrial tissue sampling on relative expression of PPARδ (A), PPARγ1 (B), PPARγ2 (C), ANGPTL4 (D), and ADD1 (E) mRNA in endometrial tissue. Sows were from Duroc×Yorkshire-Landrace breed. Data are ratios of selected genes relative mRNA levels normalized to cyclophilin housekeeping gene mRNA levels. At site, endometrial tissue sample taken at embryo attachment sites; Intersite, endometrial tissue sample taken between attachment sites. Each bar represents least square means ± S.E.M.; *P < 0.05.
in pigs (Grindflek et al. 1998, Sundvold et al. 2001), no studies have yet reported a possible involvement of these receptors in pig pregnancy. In the current study, the presence of PPAR\(\delta\) and \(\gamma\) transcripts and proteins in pig endometrium, along with modulation of their mRNA abundance according to the parity, pregnancy stage, and site of endometrial tissue sampling, suggest a possible role of these PPARs in early pig gestation. We have shown by immunohistochemistry that PPAR\(\delta\) and \(\gamma\) proteins are mainly localized in epithelial and glandular cells of the pig endometrium. The principal difference between the PPAR\(\delta\) and \(\gamma\) signals was the decrease or loss of protein expression in endometrial glands at day 25 compared with day 15 of pregnancy. Moreover, the presence of trophoblast was associated with the detection of PPAR\(\delta\) and \(\gamma\) signals in the subepithelial stroma at attachment but not between sites, and this was only seen in day 15 pregnant sows. Expression of PPAR\(\delta\) was also reported in the subluminal stroma at implantation sites in mouse (Lim et al. 1999) and rat (Ding et al. 2003a) uterus, where immunostaining signals were much stronger. Our data suggest that PPAR\(\delta\) and \(\gamma\) protein-specific expression in subepithelial stroma is stimulated by the presence of trophoblast during the periimplantation period (day 15) since no corresponding signals were detected in cycling or in day 25 pregnant sows. We have also shown that day 25 trophoblast expresses both PPAR\(\delta\) and \(\gamma\) proteins. Previous reports demonstrated that PPAR\(\gamma\) is required for epithelial differentiation of mouse trophoblast (Barak et al. 1999),

![Figure 7](https://www.reproduction-online.org/reproduction/2006/131/929-942/fig7.png)
and strong PPARγ immunostaining was also observed in bovine trophoderm and inner cell mass cells of preattachment embryos (Mohan et al. 2002). PPARδ is also a critical mediator of embryo implantation since its deficiency is lethal to over 90% of mice embryos (Barak et al. 2002). Thus, the presence of PPARδ and γ immunostaining in endometrial and trophoblast cells suggests that these nuclear receptors play an important role during early pregnancy in sows.

Our results have shown that pig PPARγ2 mRNA levels were not affected by parity, pregnancy stage, or site of endometrial tissue sampling. PPARγ2 is strongly up-regulated during adipogenesis (Tontonoz et al. 1994) and is able to completely restore adipogenesis in 3T3-L1 cells in which both PPARγ1 and γ2 expression had been abolished, whereas PPARγ1 had no effect (Ren et al. 2002). In contrast, Barak et al. (1999) reported elevated expression of PPARγ1, but not PPARγ2 mRNA, in mouse placenta from embryonic day 8.5 and onwards, thus suggesting a possible role of PPARγ1, but not PPARγ2, during early pregnancy. In accordance with this later study, we see a marked increase of PPARγ1 mRNA, but not of PPARγ2, in endometrial tissues of day 25 compared to day 15 pregnant sows. We could not confirm this difference at the protein level due to lack of isoform-specific antibodies.

Our results also show that PPARγ1 and PPARδ mRNA abundance was lower at attachment sites relative to regions between sites, for both day 15 and day 25 pregnant sows. Although the role of PPARγ in early pregnancy is not known in its entirety, recent studies have shown that PPARγ ligands can have anti-angiogenic actions, as demonstrated by the inhibition of endothelial proliferation and differentiation in vitro and suppression of VEGF-induced angiogenesis in vivo (Xin et al. 1999, Panigraphy et al. 2002). It is worth noting that ANGPTL4 mRNA levels were similarly reduced at pig embryo attachment sites in both day 15 and day 25 pregnant sows. ANGPTL4, which is a known downstream target of PPARγ, was also reported to inhibit angiogenesis and vascular permeability both in vitro and in vivo (Ito et al. 2003). Thus, a down-regulation of anti-angiogenic factors such as PPARγ1 and ANGPTL4 at the sites of attachment may result in increased uterine vascular permeability and angiogenesis, both required for successful trophoblast–endometrial epithelium interaction in pigs. However, an anti-angiogenic role for PPARγ1 and ANGPTL4 in pig early pregnancy remains to be demonstrated, as PPARγ and ANGPTL4 have also been described as potent proangiogenic factors (Yamakawa et al. 2000, Le Jan et al. 2003). The decreased mRNA levels of PPARδ at the site of implantation remain to be explained as this receptor has been described as a mediator of cell proliferation and/or angiogenesis at implantation sites in mice (Lim et al. 1999).

The role of PPARδ in embryo implantation was demonstrated in COX-2-deficient mice (Lim et al. 1999) where the COX-2-derived prostaglandin I2 (PGI2) participates in implantation via the nuclear receptor PPARδ in the mouse uterus. In the current study, the lower mRNA abundance of PPARδ found in day 15 pregnant sows when compared with day 15 cyclic sows suggests a limited role of PPARδ during the peri-attachment period. Previous studies have reported higher PGE2 in uterine fluid of day 15 pregnant gilts when compared to the day 15 cyclic gilts (Chabot et al. 2004) and higher PGF2α and PGE2 levels in uterine flushings of pregnant relative to non-pregnant gilts (Geisert et al. 1982). Thus, the elevated levels of prostaglandins (Chabot et al. 2004) and the lower mRNA abundance of PPARδ (current study) in day 15 pregnant sows suggest that PPARδ may be of modest significance in the mediation of action of endometrial and conceptus-derived prostaglandins during the attachment period. Alternatively, the higher level of PPARδ mRNA found in cyclic sows suggests a putative role for PPARδ during the estrous cycle. Thus, it will be of interest to study further the expression of PPARδ relative to the expression of COX-2 and of PGF2α and PGE2 through the estrous cycle and pregnancy.

In this study, we report lower levels of PPARδ, ANGPTL4, and ADD1 mRNA in nulliparous relative to multiparous YL sows. There are numerous examples of parity-based differences in livestock reproductive performance but evidence of physiological mechanisms to explain them are still lacking. We previously reported larger litter size and a greater number of corpora lutea (CL) in multiparous than in YLn sows (Guay et al. 2001). Moreover, higher uterine content of estradiol-17 β (E2) along with higher endometrial mRNA levels of COX-2 were found in multiparous sows compared to nulliparous ones (Guay et al. 2004). Interestingly, estrogen treatment can induce PPARδ expression in rat glandular epithelium (Ding et al. 2003a) and expression of PPARγ was also induced by E2 in zebrafish hepatocytes (Ibabe et al. 2005). Moreover, Nunez et al. (1997) reported that PPARs are capable of activating estrogen-responsive genes in the CV-1 tumor cell line. Thus, the higher levels of PPARδ mRNA found in multiparous sows may be explained by a higher E2 uterine content, as previously reported for multiparous sows (Guay et al. 2004).

This is the first report of ANGPTL4 and ADD1 mRNA in endometrial tissue, and the relative elevation of these transcripts in multiparous sows remains to be explained. Adipocyte determination differentiation-dependent factor 1 (ADD1) is an intracellular membrane-bound transcription factor (Golgi and endoplasmic reticulum) that controls the metabolism of cholesterol and fatty acids in animal cells (Wang et al. 1994, Kim & Spiegelman 1996). Interestingly, it was also reported that ADD1 controls the production of endogenous ligands for PPARγ in NIH 3T3 cells (Kim et al. 1999). The detection of ADD1 transcript in endometrial tissue may illustrate that PPAR ligands are also produced.
endogenously from endometrial cells expressing ADD1. Because ADD1 would provide PPAR ligands, it is not surprising that these transcripts follow the same expression profile in pig endometrial tissue. However, since ADD1 mRNA abundance was affected by neither pregnancy stage nor site of endometrial tissue sampling, we believe that this transcription factor has a limited role in early porcine gestation.

In summary, we have demonstrated that PPARδ, PPARγ1, and ANGPTL4 mRNA abundance decreases at the embryonic attachment sites in the pig endometrium at both day 15 and day 25 of pregnancy. Modulation of PPARγ1 expression was also observed according to stage of pregnancy. The parity of sows also had effects on PPARδ, ANGPTL4, and ADD1 transcripts. The mRNA abundance of PPARγ2 was not affected by the site of endometrial tissue sampling, pregnancy stage, or parity of the sows, thus suggesting that the PPARγ2 subtype does not have a major role in porcine pregnancy. Because this study was performed on a limited number of pigs, further work will be needed on a much larger population and at various stages of pregnancy to confirm the current findings. The precise role of PPARδ, PPARγ1, and ANGPTL4 genes in early pig pregnancy remains to be established, but our findings suggest that they may have key roles in prostaglandins-mediated action on the endometrial tissue.

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References


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