

Expression and regulation of prostaglandin transporters, ATP-binding cassette, subfamily C, member 1 and 9, and solute carrier organic anion transporter family, member 2A1 and 5A1 in the uterine endometrium during the estrous cycle and pregnancy in pigs

Hwanhee Jang¹, Yohan Choi¹, Inkyu Yoo¹, Jisoo Han¹, Minjeong Kim¹, and Hakhyun Ka^{1,*}

* **Corresponding Author:** Hakhyun Ka
Tel: +82-33-760-2369, **Fax:** +82-33-760-2183,
E-mail: hka@yonsei.ac.kr

¹Department of Biological Science and Technology,
Yonsei University, Wonju 26493, Korea

Submitted Aug 22, 2016; Revised Sept 13, 2016;
Accepted Oct 3, 2016

Objective: Prostaglandins (PGs) function in various reproductive processes, including luteolysis, maternal pregnancy recognition, conceptus development, and parturition. Our earlier study has shown that PG transporters ATP-binding cassette, subfamily C, member 4 (*ABCC4*) and solute carrier organic anion transporter family, member 2A1 (*SLCO2A1*) are expressed in the uterine endometrium in pigs. Since several other PG transporters such as *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* are known to be present in the uterine endometrium, this study investigated the expression of these PG transporters in the porcine uterine endometrium and placenta.

Methods: Uterine endometrial tissues were obtained from gilts on day (D) 12 and D15 of the estrous cycle and days 12, 15, 30, 60, 90, and 114 of pregnancy.

Results: *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs were expressed in the uterine endometrium, and levels of expression changed during the estrous cycle and pregnancy. Expression of *ABCC1* and *ABCC9* mRNAs was localized mainly to luminal and glandular epithelial cells in the uterine endometrium, and chorionic epithelial cells during pregnancy. Conceptuses during early pregnancy and chorioallantoic tissues from mid to late pregnancy also expressed these PG transporters. Estradiol-17 β increased the expression of *ABCC1* and *SLCO5A1*, but not *ABCC9* and *SLCO4C1* mRNAs and increasing doses of interleukin-1 β induced the expression of *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs in endometrial explant tissues.

Conclusion: These data showed that several PG transporters such as *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* were expressed at the maternal-conceptus interface, suggesting that these PG transporters may play an important role in the establishment and maintenance of pregnancy by regulating PG transport in the uterine endometrium and placenta in pigs.

Keywords: Pig; Uterus; Endometrium; Prostaglandin; Prostaglandin Transporters

INTRODUCTION

Prostaglandins (PGs) are derived from arachidonic acid, a polyunsaturated fatty acid in the cell membrane phospholipids, by sequential enzymatic actions of cyclooxygenase 1 and 2 and other intracellular enzymes [1]. PGs play key roles in many reproductive processes such as luteolysis, implantation, conceptus development and parturition in many mammalian species [2,3]. Among the various PGs, it has been shown that PGE₂ and PGF_{2 α} are involved in conceptus implantation in pigs [4,5]. In pigs, the conceptuses undergo dramatic morphological changes from spherical to filamentous form and secrete hormones and cytokines during the implantation period [4]. Estrogen secreted by the elongating conceptus acts as a maternal recognition signal by changing the direction of luteolytic PGF_{2 α} secretion from the uterine blood vessel to the uterine lumen.

Thus, $\text{PGF}_{2\alpha}$ does not move to the ovary, but exerts luteolytic action on the corpus luteum (CL), so that CL can remain and secrete progesterone (P_4) for maintenance of pregnancy [4]. It has been shown that PGE_2 is produced by the implanting conceptuses and endometrium at the time of implantation [6,7]. PGE_2 has a luteotrophic effect in the ovary [5].

Because PGs are lipid-derived hormones bearing negative charge, PGs cannot pass through the plasma membrane easily. Thus, PG transporters are required to mediate the transport of PGs through the plasma membrane [1]. In our earlier study, we have shown that PG transporters such as ATP-binding cassette, subfamily C, member 4 (*ABCC4*; also known as multidrug resistance protein 4 [*MRP4*]) and solute carrier organic anion transporter family, member 2A1 (*SLCO2A1*, also known as PG transporter [*PGT*] or organic anion-transporting polypeptide 2A1 [*OATP2A1*]), are expressed in the uterine endometrium during early pregnancy in pigs [8]. *ABCC4* and *SLCO2A1* mRNAs are expressed in a stage and cell type specific manner in the uterine endometrium during pregnancy, and their expression is increased by interleukin- 1β (IL1B) during the implantation period [8]. In addition to *ABCC4* and *SLCO2A1*, many other members of the ABCs and SLCO family are known to be involved in PG transport through the cell membrane [9]. Among those, we selected, for the microarray and RNA-seq analyses, four PG transporters, *ABCC1* (also known as *MRP1*), *ABCC9* (also known as sulfonylurea receptor 2 [*SUR2*]), *SLCO4C1* (also known as *OATP4C1*), and *SLCO5A1* (also known as *OATP5A1*), because these have been shown to be differentially expressed in the uterine endometrium during early pregnancy [10,11].

ABCC1 expression has been shown in many species, and in human, *ABCC1* mRNA is expressed in various tissues including placenta, liver, and kidney [12,13]. *ABCC9* is a subunit of ATP-sensitive K^+ channel and is expressed in many tissues including myometrium, cardiac and skeletal muscle [14]. *SLCO4C1* and *SLCO5A1* are also expressed in many tissues including kidney, liver, and brain [15,16]. In addition to transporting PG, these transporters are also involved in the transport of various molecules, including organic anions, amphipathic organic compounds, and drugs [17]. Although these transporters are known to mediate transport of various molecules including PGs in many tissues, expression and function of these PG transporters in the establishment and maintenance of pregnancy have not been determined.

During the implantation period in pigs, the elongating conceptuses produce estrogen and IL1B2. Estrogen acts as a signal for maternal recognition of pregnancy. These conceptus-derived factors induce expression of many endometrial genes [4]. Although it has been shown that expression of *ABCC1* gene is induced by estrogen and progesterone in human trophoblast cells [18], regulatory mechanisms of the expression of PG transporter genes, *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* are not well understood.

Therefore, to understand the expression and regulation of PG transporters in porcine uterine endometrium during the estrous cycle and pregnancy, we evaluated i) expression of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs in the uterine endometrium during the estrous cycle and pregnancy, in the conceptus of early pregnancy, and in chorioallantoic tissues during pregnancy; ii) localization of *ABCC1* and *ABCC9* mRNAs in the uterine endometrium; and iii) effects of steroid hormones, IL1B, and interferon gamma (IFNG) on *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNA expression in the endometrial tissues.

MATERIALS AND METHODS

Animals and tissue preparation

All experimental procedures involving animals were conducted in accordance with the Guide for Care and Use of Research Animals in Teaching and Research and approved by the Institutional Animal Care and Use Committee of Yonsei University. Sexually mature crossbred female gilts were assigned randomly to either cyclic or pregnant status. Gilts were observed for estrous behavior daily and artificially inseminated at the onset of estrus (day [D] 0) and 24 h later with fresh boar semen. The reproductive tracts of gilts were obtained immediately after they were slaughtered at a local slaughterhouse on either D 12 or D15 of the estrous cycle and on D12, D15, D30, D60, D90, or D114 of pregnancy (n = 3 to 6 gilts/d/status). Pregnancy was confirmed by the presence of apparently normal filamentous conceptuses in uterine flushings on D12 and D15 and presence of embryos and placenta in the later days of pregnancy. Chorioallantoic tissues were obtained from D30, D60, D90, and D114 of pregnancy (n = 3 to 4/d).

Endometrium, dissected free of myometrium, was collected from the middle portion of each uterine horn, snap-frozen in liquid nitrogen, and stored at -80°C prior to RNA extraction. For *in situ* hybridization, cross-sections of endometrium were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 24 h and then embedded in paraffin as previously described [8].

Total RNA extraction and cloning of porcine *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* cDNAs

Total RNA was extracted from endometrial, chorioallantoic, and conceptus tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The quantity of RNA was assessed spectrophotometrically, and integrity of RNA was validated by electrophoresis in 1% agarose gel.

Four micrograms each of total RNA from endometrial tissues and 1 μg each from conceptus tissues were treated with DNase I (Promega, Madison, WI, USA) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen, USA) to obtain cDNAs. The cDNA templates were then diluted 1:4 with nuclease-

free water and amplified by polymerase chain reaction (PCR) using Taq polymerase (Takara Bio, Shiga, Japan), and specific primers based on porcine *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNA sequences. The PCR conditions, sequences of the primer pairs, and expected product sizes are listed in Table 1. The PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining. The identity of each amplified PCR product was verified by sequence analysis after cloning into the pCRII vector (Invitrogen, USA).

Quantitative real-time reverse transcription-PCR

The levels of expression of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* genes in endometrial and chorioallantoic tissues were analyzed by real-time RT-PCR using the Applied Biosystems StepOnePlus System (Applied Biosystems, Foster City, CA, USA) and SYBR Green method. cDNAs were synthesized from 4 µg each of the total RNA isolated from different uterine endometrial tissues. Newly synthesized cDNAs (total volume of 21 µL) were diluted 1:4 with sterile water and then used for PCR. The Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used for PCR reactions. The final reaction volume of 20 µL included 2 µL of cDNA, 10 µL of 2X Master mix, 2 µL of each primer, and 4 µL of double distilled water. PCR conditions and sequences of primer pairs are listed in Table 1. Results are reported as expression relative to that detected on D12 of the estrous cycle or that detected in control explant tissues after normalization of the transcript amount to the endogenous ribosomal protein L7 (*RPL7*) control by the $2^{-\Delta\Delta CT}$ method as previously described [19].

Non-radioactive *in situ* hybridization

Non-radioactive *in situ* hybridization was performed to deter-

mine the localization of *ABCC1* and *ABCC9* expression in the uterine endometrium as previously described with some modifications [20]. Sections (5 µm thick) were rehydrated by immersion in successive baths of xylene, 100% ethanol, 95% ethanol, diethylpyrocarbonate (DEPC) treated water, and DEPC treated PBS. Tissue sections were boiled in citrate buffer (pH 6.0) for 10 min. After washing in DEPC treated PBS, they were digested using 5 µg/mL proteinase K (Sigma, St. Louis, MO, USA) in Tris-ethylenediaminetetraacetic acid (EDTA) buffer (100 mM Tris-HCl, 50 mM EDTA, pH 7.5) buffer at 37°C. After post-fixation in 4% paraformaldehyde, tissue sections were incubated twice, for 15 min each, in PBS containing 0.1% active DEPC and equilibrated for 15 min in 5× sodium chloride-sodium citrate (SSC) [21]. The sections were pre-hybridized for 2 h at 68°C in the hybridization mix (50% formamide, 5× SSC, 500 µg/mL herring sperm DNA, 250 µg/mL yeast tRNA). Sense and antisense riboprobes for each gene were generated using partial cDNAs cloned into pCRII vectors by linearizing with appropriate restriction enzymes and labeling with digoxigenin (DIG)-UTP using a DIG RNA Labeling kit (Roche, Indianapolis, IN, USA). The probes were denatured for 5 min at 80°C and added to the hybridization mix. The hybridization reaction was carried out overnight at 68°C. Pre-hybridization and hybridization reactions were performed in a box saturated with 5× SSC-50% formamide solution to avoid evaporation, and no coverslips were used. After hybridization, sections were washed for 30 min in 2× SSC at room temperature, 1 h in 2× SSC at 65°C, and 1 h in 0.1× SSC at 65°C. Probes bound to the sections were detected immunologically, using sheep anti-DIG Fab fragments covalently coupled to alkaline phosphatase and nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (toluidine salt) as the chromogenic substrate, according to the manufacturer's protocol (Roche, USA).

Table 1. Summary of PCR primer sequences and expected product sizes

Primer	Sequence of forward (F) and reverse (R) primers (5'→3')	Annealing temperature (°C)	Product size (bp)	No. of cycles	GenBank accession no.
For <i>in situ</i> hybridization					
<i>ABCC1</i>	F: AGA CCT GGA CCT GGT TCT CC R: CGT CCA GAC CTC TTC CTC TG	54	292	40	XM_005662115.1
<i>ABCC9</i>	F: ACC TGT TGG CGA TAC CTC AC R: GAC AAG GCA GAG GAA AAT GC	54	216	40	XM_005655658.1
For RT-PCR and/or real-time RT-PCR					
<i>ABCC1</i>	F: AGT TTA TTC CCA CTT CAA CGA GAC R: TGC AGT GAG TAA GAC ACT GAG AGG	60	267	40	XM_005662115.1
<i>ABCC9</i>	F: AAG ATA TCA CCT GGA CAG CTA TGA G R: AGG TTC AGA TTC ATT TAC ATT GCT C	60	274	40	XM_005655658.1
<i>SLCO4C1</i>	F: TTT ACC AGG TAC AGA AGA AAT TCA AG R: ATT TAG GTA AAA ATG TGG CAA ATC C	60	215	40	XM_003480882.2
<i>SLCO5A1</i>	F: CTG TTC ATA GTG ACC TTC ATC ACA G R: TAA AAA TAA AGC CAA CGA ATT TGA G	60	295	40	XM_005663031.1
<i>RPL7</i>	F: AAG CCA AGC ACT ATC ACA AGG AAT ACA R: TGC AAC ACC TTT CTG ACC TTT GG	60	177	40	NM_001113217

PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; *ABCC1*, ATP-binding cassette, subfamily C, member 1; *ABCC9*, ATP-binding cassette, subfamily C, member 9; *SLCO4C1*, solute carrier organic anion transporter family, member 4C1; *SLCO5A1*, solute carrier organic anion transporter family, member 5A1; *RPL7*, ribosomal protein L7.

Explant cultures

To determine the effect of steroid hormones, estradiol-17 β (E₂) and P₄, and IL1B on the expression of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs in the uterine endometrium, endometrial explant tissues obtained from gilts on D12 of the estrous cycle were cultured as previously described [8]. Endometrium on D12 of the estrous cycle was dissected from the myometrium and placed into warm, phenol red free Dulbecco's modified Eagle's medium/F-12 culture medium (DMEM/F-12; Sigma, USA) containing penicillin G (100 IU/mL) and streptomycin (0.1 mg/mL). The endometrium was minced with scalpel into small pieces (2 to 3 mm³), and aliquots of 500 mg were placed into T25 flasks with serum free modified DMEM/F-12 containing 10 μ g/mL insulin (Sigma, USA), 10 ng/mL transferrin (Sigma, USA), and 10 ng/mL hydrocortisone (Sigma, USA). Endometrial explants were cultured immediately after mincing in the presence of ethanol (control), E₂ (50 ng/mL; Sigma, USA), P₄ (3 ng/mL; Sigma, USA), P₄+E₂, P₄+E₂+ICI182,780 (ICI; an estrogen receptor antagonist; 200 ng/mL; Tocris Bioscience, Ellisville, MO, USA), or P₄+E₂+RU486 (RU, a progesterone receptor antagonist; 30 ng/mL; Sigma, USA) for 24 h with shaking in an atmosphere of 5% CO₂ in air at 37°C. To determine the effects of IL1B on expression of endometrial genes, explant tissues were treated with 0, 1, 10, or 100 ng/mL IL1B (Sigma, USA) in the presence of both E₂ (50 ng/mL) and P₄ (3 ng/mL) at 37°C for 24 h. Explant tissues were then collected and total RNA or protein was extracted for real-time RT-PCR and immunoblot analysis, respectively, to determine the expression levels of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs. These experiments were conducted using endometrium on D12 of the estrous cycle, and treatments were performed in triplicate using tissues obtained from three gilts.

Statistical analysis

Data from real-time RT-PCR for *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* expression during the estrous cycle and pregnancy were subjected to least squares analysis of variance using the general linear models procedures of SAS (Cary, NC, USA). As sources of variation, the model included day, pregnancy status (cyclic or pregnant, D12 and D15 post-estrus), and their interactions, to evaluate steady-state levels of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs, and effects of treatment and animal, to evaluate effects of steroid hormones on *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs. Preplanned orthogonal contrasts (control vs E₂; control vs P₄; E₂ vs E₂+P₄; E₂+P₄ vs E₂+P₄+ICI; and E₂+P₄ vs E₂+P₄+RU) were used to test for effects of treatments in the explant cultures. Data from real-time RT-PCR performed to assess the effect of the day of pregnancy (D12, D15, D30, D60, D90, and D114) in the endometrium, effect of the day of pregnancy (D30, D60, D90, and D114) in chorioallantoic tissue, on the expression of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1*, and data from IL1B dose-response studies were analyzed by least squares regression analysis. Data are presented as least squares

means with SEM. A p-value less than 0.05 was considered significant, whereas a p-value of 0.05 to 0.10 was considered a trend toward significance.

RESULTS

Expression of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs in the uterine endometrium during the estrous cycle and pregnancy in pigs

To determine the expression of mRNAs for PG transporters *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* in the porcine uterine endometrium during the estrous cycle and pregnancy, we performed real-time RT-PCR analysis (Figure 1). On D12 and D15 post-estrus, expression of *ABCC1* mRNA was affected by pregnancy status (p<0.01) and day \times status (p<0.05), but not by day (Figure 1A). Levels of *ABCC9* and *SLCO4C1* mRNAs were affected by pregnancy status (p<0.05), but not by day or day \times status (Figure 1B, 1C). Levels of *SLCO5A1* mRNA were affected by pregnancy status, day, and day \times status (p<0.05) (Figure 1D). Expression of *ABCC1* and *SLCO5A1* mRNAs was greater on D12 of pregnancy than on D12 of the estrous cycle. Steady-state levels of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs changed during pregnancy with the highest levels on D30 for *ABCC1* and *ABCC9*, on D90 for *SLCO4C1*, and on D12 for *SLCO5A1* (cubic effect of day for *ABCC1*, p<0.01; linear effect of day for *ABCC9*, p<0.05; cubic effect of day for *SLCO4C1*, p<0.05; linear effect of day for *SLCO5A1*, p = 0.788) (Figure 1A-1D).

Expression of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs in conceptuses during early pregnancy and in chorioallantoic tissues during late pregnancy

Next, to determine if *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs were expressed in conceptuses during early pregnancy, we performed RT-PCR using cDNAs from conceptuses from D12 and D15 of pregnancy. As shown in Figure 2A, *ABCC1*, *ABCC9*, and *SLCO4C1* mRNAs were detected in conceptus on D12 of pregnancy, and *SLCO5A1* mRNA was detected in conceptus on D12, but not on D15 of pregnancy. We also performed real-time RT-PCR to determine if levels of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs change in chorioallantoic tissues during mid to late pregnancy. As shown in Figure 2B, levels of *ABCC1* and *ABCC9*, but not *SLCO4C1* and *SLCO5A1* mRNAs changed in chorioallantoic tissues on D30, D60, D90, and D114 of pregnancy (quadratic effect of day for *ABCC1*, p = 0.075; linear effect of day for *ABCC9*, p<0.05).

Localization of *ABCC1* and *ABCC9* mRNAs in the uterine endometrium during the estrous cycle and pregnancy in pigs

To determine the cell type(s) that express *ABCC1* and *ABCC9* mRNAs in the uterine endometrium, we performed *in situ* hybridization analysis. As shown in Figure 3, both *ABCC1* and *ABCC9* mRNAs were detected mainly in luminal (LE) and glandular

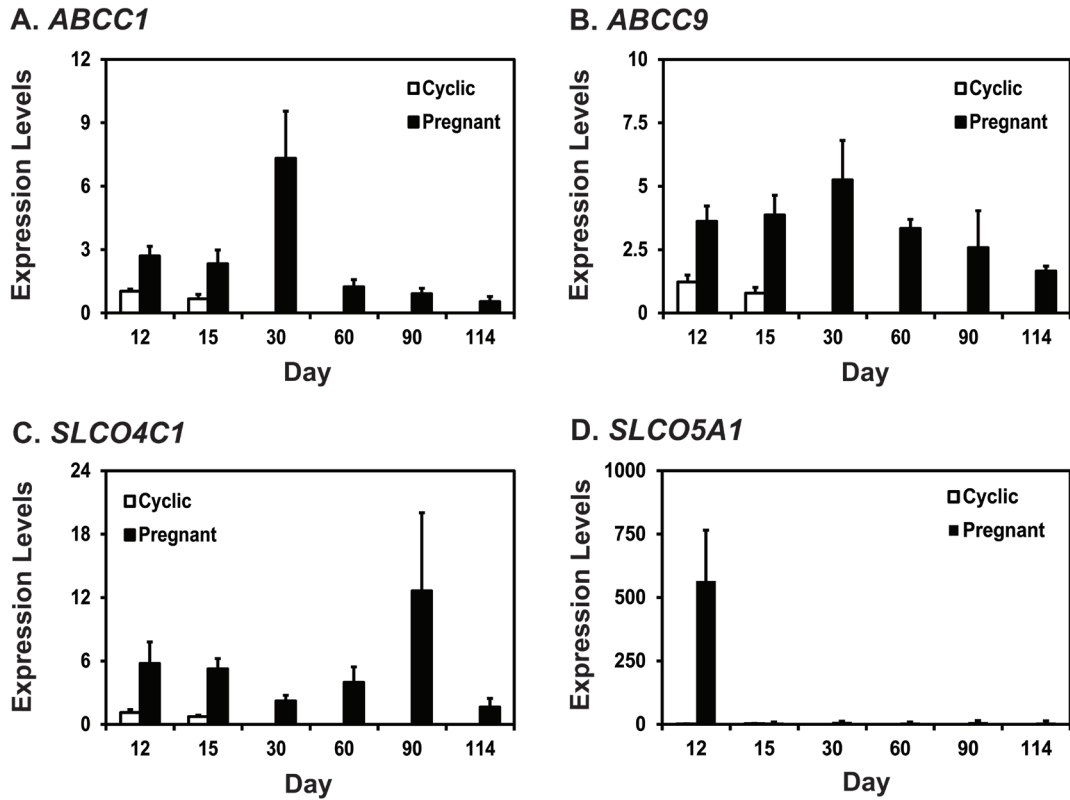


Figure 1. Expression of *ABCC1* (A), *ABCC9* (B), *SLCO4C1* (C), and *SLCO5A1* (D) mRNAs in porcine uterine endometria during the estrous cycle and pregnancy. Endometrial tissue samples from cyclic and pregnant gilts were analyzed by real-time RT-PCR and data are reported as expression relative to that detected on day (D) 12 of the estrous cycle after normalization of the transcript amount to the endogenous *RPL7* control. Data are presented as least squares means with standard error. *ABCC1*, ATP-binding cassette, subfamily C, member 1; *ABCC9*, ATP-binding cassette, subfamily C, member 9; *SLCO4C1*, solute carrier organic anion transporter family, member 4C1; *SLCO5A1*, solute carrier organic anion transporter family, member 5A1; RT-PCR, reverse-transcription polymerase chain reaction; *RPL7*, ribosomal protein L7.

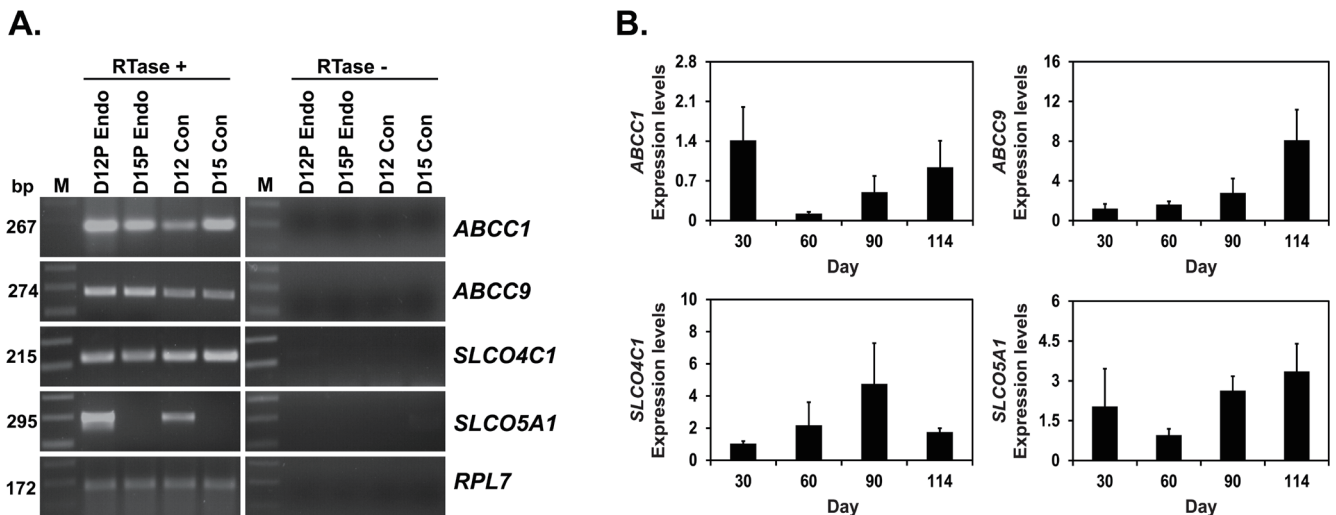


Figure 2. Expression of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs by conceptuses from day 12 and day 15 of pregnancy and by chorioallantoic tissues during the later stages of pregnancy. (A) RT-PCR analysis of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs in conceptuses on day (D) 12 and D15 of pregnancy was done using total RNA preparations. *RPL7* was used as a positive control. RTase +/-, with (+) or without (-) reverse transcriptase; M, molecular weight marker; D12 Endo, endometrium on D12 of pregnancy; D12 Con, D12 conceptus; D15 Con, D15 conceptus. (B) Real-time RT-PCR analysis of the expression of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs in chorioallantoic tissue samples on D30, D60, D90, and D114 of pregnancy. Data are reported as expression relative to that detected on D30 of pregnancy after normalization of the transcript amount to the endogenous *RPL7* control, and data are presented as least squares means with standard errors. *ABCC1*, ATP-binding cassette, subfamily C, member 1; *ABCC9*, ATP-binding cassette, subfamily C, member 9; *SLCO4C1*, solute carrier organic anion transporter family, member 4C1; *SLCO5A1*, solute carrier organic anion transporter family, member 5A1; RT-PCR, reverse-transcription polymerase chain reaction; *RPL7*, ribosomal protein L7.

epithelial (GE) cells in the endometrium, and also in chorionic epithelial (CE) and stromal cells in the chorioallantoic tissues (Figure 3A, 3B). Localization of *SLCO4C1* and *SLCO5A1* mRNAs could not be done, because the expression levels of *SLCO4C1* and *SLCO5A1* mRNAs in the tissues were too low and signal intensity was very weak.

Effects of steroid hormones, E₂ and P₄, and IL1B on *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNA expression in the uterine endometrium

Since the levels of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs were significantly higher on D12 of pregnancy than those during the estrous cycle and E₂ and IL1B from conceptus and/or P₄ from corpus luteum (CL) regulate expression of many uterine genes in the endometrium during the peri-implantation period, we examined if E₂, P₄, and/or IL1B affect the expression

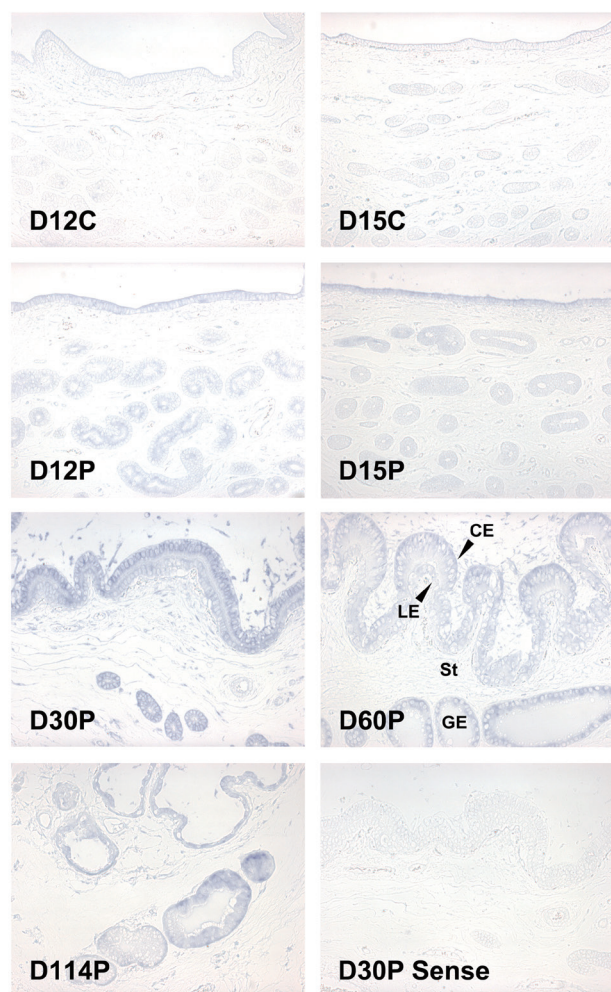
of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs in endometrial explant tissues from D12 of the estrous cycle (Figures 4, 5).

As shown in Figure 4, *ABCC1* and *SLCO5A1* mRNA levels were increased by E₂ (control vs E₂, p<0.05 for *ABCC1* and *SLCO5A1*; P₄ vs P₄+E₂, p<0.05 for *ABCC1* and p = 0.064 for *SLCO5A1*). However, levels of *ABCC9* and *SLCO5C1* mRNAs were not affected by E₂ and P₄. As shown in Figure 5, IL1B did not affect the levels of *ABCC1* and *ABCC9* mRNAs, but increased the levels of *SLCO4C1* and *SLCO5A1* mRNAs in the endometrial tissues (quadratic effect of dose for *SLCO4C1*, p<0.05; linear effect of dose for *SLCO5A1*, p<0.01).

DISCUSSION

The significant findings of this study are: i) *ABCC1*, *ABCC9*, *SL-*

A. *ABCC1*



B. *ABCC9*

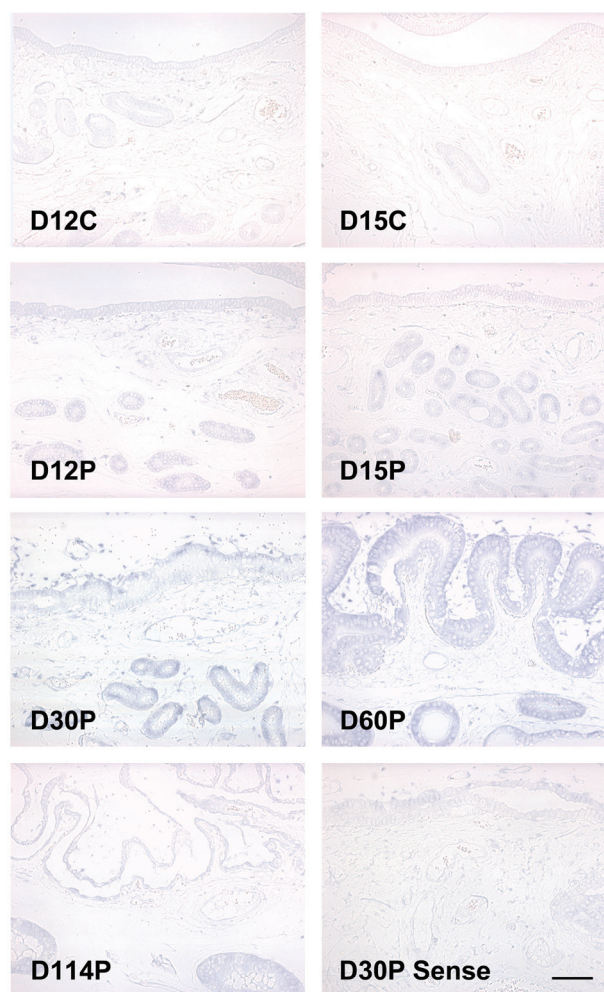


Figure 3. *In situ* hybridization analysis of *ABCC1* (A) and *ABCC9* (B) mRNAs in the uterine endometrium during the estrous cycle and pregnancy in pigs. Representative uterine sections from day 12 of pregnancy, hybridized with a digoxigenin-labeled sense *ABCC1* or *ABCC9* cRNA probe (sense) as a negative control, are shown. *ABCC1*, ATP-binding cassette, subfamily C, member 1; *ABCC9*, ATP-binding cassette, subfamily C, member 9; D, day; C, estrous cycle; P, pregnancy; LE, luminal epithelium; GE, glandular epithelium; St, stroma; CM, chorionic membrane. Bars = 100 μ m.

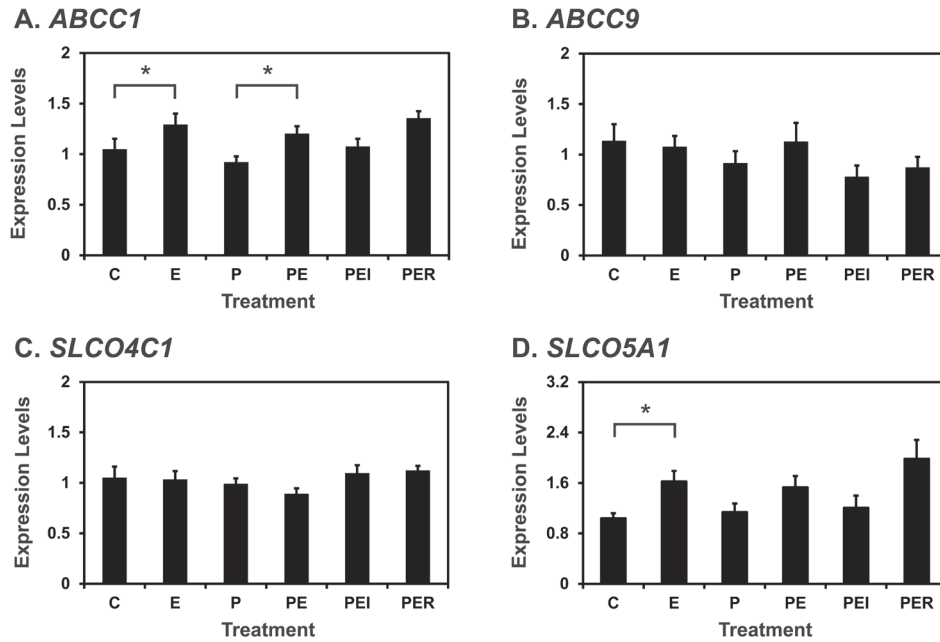


Figure 4. Effect of steroid hormones on the expression of *ABCC1* (A), *ABCC9* (B), *SLCO4C1* (C), and *SLCO5A1* (D) mRNAs in endometrial explant cultures. Endometrial explants from gilts on day 12 of the estrous cycle were cultured in DMEM/F-12 in the presence of control (C), E₂ (E), P₄ (P), E₂+P₄ (PE), E₂+P₄+ICI (I, an estrogen receptor antagonist) (PEI), or E₂+P₄+RU (R, a progesterone receptor antagonist) (PER) at 37°C for 24 h. All the experiments were done in triplicate with endometrium from three gilts for each treatment. Level of mRNA expression based on real-time RT-PCR analysis is relative to that for *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs in the control group of endometrial explants after normalization of transcript amounts to *RPL7* mRNA. Data are presented as least squares means with standard errors. *ABCC1*, ATP-binding cassette, subfamily C, member 1; *ABCC9*, ATP-binding cassette, subfamily C, member 9; *SLCO4C1*, solute carrier organic anion transporter family, member 4C1; *SLCO5A1*, solute carrier organic anion transporter family, member 5A1; DMEM/F-12, Dulbecco's modified Eagle medium/F-12; RT-PCR, reverse-transcription polymerase chain reaction. * p<0.05.

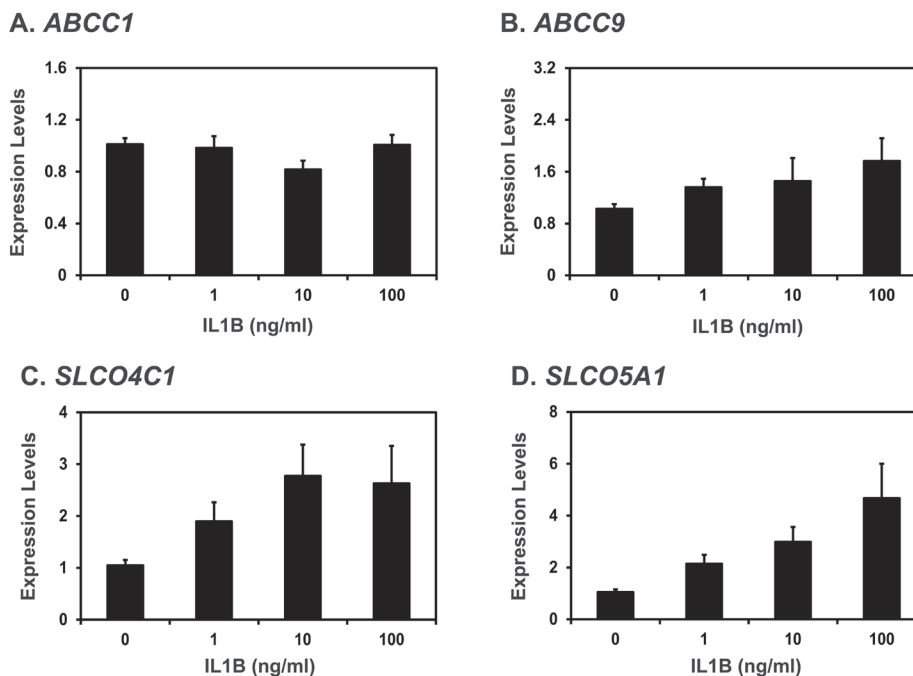


Figure 5. Effect of IL1B on *ABCC1* (A), *ABCC9* (B), *SLCO4C1* (C), and *SLCO5A1* (D) mRNA levels in endometrial explant cultures. Endometrial explants from gilts on day 12 of the estrous cycle were cultured in DMEM/F-12 with 0, 1, 10, 100 ng/mL IL1B in the presence of both E₂ (50 ng/mL) and P₄ (3 ng/mL), at 37°C for 24 h. All experiments were done in triplicate with endometrium from three gilts for each treatment. Level of mRNA expression determined by real-time RT-PCR analysis is relative to that for *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs in the control group of endometrial explants (0 ng/mL IL1B) after normalization of transcript amounts to *RPL7* mRNA. Data are presented as least squares means with standard errors. IL1B, interleukin-1β; *ABCC1*, ATP-binding cassette, subfamily C, member 1; *ABCC9*, ATP-binding cassette, subfamily C, member 9; *SLCO4C1*, solute carrier organic anion transporter family, member 4C1; *SLCO5A1*, solute carrier organic anion transporter family, member 5A1; DMEM/F-12, Dulbecco's modified Eagle medium/F-12; RT-PCR, reverse-transcription polymerase chain reaction; *RPL7*, ribosomal protein L7.

CO4C1, and *SLCO5A1* mRNAs are expressed in the uterine endometrium in pregnancy status and stage specific manner; ii) *ABCC1* and *ABCC9* mRNAs are localized mainly to LE, GE, and CE cells during mid to late pregnancy; iii) conceptus tissues on days 12 and 15 of pregnancy and chorioallantoic tissues from day 30 to term pregnancy express *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs; iv) E_2 increases the expression of *ABCC1* and *SLCO5A1* mRNAs in the endometrial tissues; and v) IL1B stimulates the expression of *SLCO4C1* and *SLCO5A1* mRNAs in the endometrial tissues.

The expression patterns of *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs during pregnancy varied from one to the other. Levels of *ABCC1* and *ABCC9* mRNAs were highest on D30 of pregnancy; levels of *SLCO4C1* mRNA were highest on D90 of pregnancy; and levels of *SLCO5A1* mRNA were highest on D12 of pregnancy. Our previous study has shown that the patterns of *ABCC4* and *SLCO2A1* expression during pregnancy were biphasic with the highest levels on D12 and D90 of pregnancy [8]. These results suggest that different types of PG transporters function to transport PG in a stage specific manner during pregnancy.

In humans, it has been shown that *ABCC1* and *ABCC9* are expressed in the amniotic and chorionic membrane [22,23]. However, to our knowledge, expression of *SLCO4C1* and *SLCO5A1* in placental tissues has not been reported so far. Results of this study showed that conceptus tissues during early pregnancy and chorioallantoic tissues during mid to late pregnancy expressed *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* mRNAs, and levels of *ABCC1* and *ABCC9* mRNAs in chorioallantoic membrane changed during mid to late pregnancy. Interestingly, *SLCO5A1* mRNA, which was expressed at the highest level in the uterine endometrium on D12 of pregnancy, was detectable in conceptus tissues on D12 of pregnancy, but not on D15 of pregnancy. Expression of *ABCC4* and *SLCO2A1* mRNA and protein has also been shown in conceptus tissues and chorioallantoic tissues [8]. These observations indicate that conceptus tissues during early pregnancy and chorioallantoic tissues during late pregnancy express PG transporters dynamically, depending on the pregnancy stage.

Localization of *ABCC1* and *ABCC9* expression at the maternal-conceptus interface during pregnancy revealed that the expression of both *ABCC1* and *ABCC9* mRNAs were detected mainly in endometrial LE and GE cells and in CE and stromal cells in chorioallantoic tissues. *ABCC1* and *ABCC9* mRNAs were expressed by the same cell types, which express *ABCC4* [8]. However, expression of *ABCC1* and *ABCC9* mRNAs was not detected in endothelial cells of endometrial blood vessels, which express *SLCO2A1* [8]. In the porcine uterine endometrium, enzymes that synthesize PG, including PG-endoperoxide synthase 1 (*PTGS1*), *PTGS2*, PGE synthase (*PTGES*), and aldo-keto reductase family 1, member b1 (*AKR1B1*), are expressed in LE and GE cells in the endometrium and CE cells in chorioallantoic

tissues during pregnancy [24,25], indicating that these cells may be the major cell types that produce PGs at the maternal-conceptus interface. Thus, PG transporters expressed by LE, GE, and CE cells may play an important role in trafficking PGs in these cell types during pregnancy. The cell types expressing *SLCO4C1* and *SLCO5A1* at the maternal-conceptus interface have not yet been determined.

Results of this study showed that levels of *ABCC1* and *SLCO2A1* mRNA were significantly higher on D12 of pregnancy than the estrous cycle and levels of *ABCC9* and *SLCO4C1* mRNA were higher during pregnancy than during the estrous cycle. In pigs, conceptus produces estrogen and IL1B2 during the implantation period [4]. The conceptus derived estrogen induces the expression of many uterine endometrial genes, including *AKR1B1*, fibroblast growth factor 7 (*FGF7*), interleukin 1 receptor accessory protein (*IL1RAP*), lysophosphatidic acid receptor 3 (*LPAR3*), secreted phosphoprotein 1 (*SPP1*), signal transducer and activator of transcription 1 (*STAT1*), and transient receptor potential vanilloid type 6 (*TRPV6*) [25-27]. The IL1B also increases the expression of many endometrial genes, including IL1B receptor genes IL1 receptor 1 (*IL1R1*) and *IL1RAP*, PG transporter genes *ABCC4* and *SLCO2A1*, a calcium binding protein gene *S100* calcium binding protein G (*S100G*), and a small lipid binding protein gene salivary lipocalin 1 (*SAL1*) [8,28]. Thus, we postulated that estrogen and/or IL1B from the conceptus might affect the expression of these PG transporters. Indeed, our explant culture study showed that E_2 induced *ABCC1* and *SLCO5A1* mRNAs, and IL1B increased *SLCO4C1* and *SLCO5A1* mRNAs in endometrial tissues, indicating that conceptus factors play critical roles in PG synthesis and transport in the uterine endometrium.

Our previous study has also shown that IL1B, but not estrogen, increases the expression of *ABCC4* and *SLCO2A1* mRNAs, which show higher levels of endometrial expression on D12 of pregnancy than during the estrous cycle in pigs [8]. An especially interesting finding of this study is that mRNAs of PG transporters *ABCC1* and *SLCO5A1* were increased by estrogen. As mentioned above, estrogen did not increase the expression of *ABCC4* and *SLCO2A1* mRNAs in our previous study [8], but that observation has raised a question as to how PG movement is regulated in estrogen-induced pseudopregnant pigs. The length of estrous cycle is extended by up to more than 30 days after treatment with estrogen between days 11 and 15 of the estrous cycle [29], and levels of plasma $PGF_{2\alpha}$ in utero-ovarian vein are low in pseudopregnant pigs [30]. Therefore, we postulated that any PG transporter(s) regulated by estrogen other than IL1B should be present and mediate PG movement into the uterine lumen in estrogen-induced pseudopregnancy. The results of the current study clearly showed that PG transporters regulated by estrogen were present in the uterine endometrium, suggesting that these transporters may be involved in the regulation of PG transport in estrogen-induced pseudopregnancy in pigs. Overall,

these results indicate that both estrogen and IL1B2 of conceptus origin may differentially regulate the expression of PG transporters in the uterine endometrium, for the establishment and maintenance of pregnancy. Regulatory mechanism of the endometrial *ABCC9* expression still needs further study.

In summary, this study showed that i) *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* genes are expressed in pregnancy stage and cell type specific manner in the uterine endometrium, ii) their expression during the implantation period is regulated by E_2 or IL1B in the endometrial tissues, and iii) conceptuses during early pregnancy and chorioallantoic tissues during mid to late pregnancy also express *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1*. Our findings suggest that *ABCC1*, *ABCC9*, *SLCO4C1*, and *SLCO5A1* play an important role in PG transport at the maternal-fetal interface for the establishment and maintenance of pregnancy in pigs.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

This study was supported by the Next Generation BioGreen 21 Program (#PJ01119103), Rural Development Administration, Republic of Korea.

REFERENCES

- Schuster VL. Molecular mechanisms of prostaglandin transport. *Annu Rev Physiol* 1998;60:221-42.
- Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and paracrine regulation of birth at term and preterm. *Endocr Rev* 2000;21:514-50.
- Spencer TE, Bazer FW. Uterine and placental factors regulating conceptus growth in domestic animals. *J Anim Sci* 2004;82 E-Suppl:E4-13.
- Bazer FW, Johnson GA. Pig blastocyst-uterine interactions. *Differentiation* 2014;87:52-65.
- Ziecik AJ. Old, new and the newest concepts of inhibition of luteolysis during early pregnancy in pig. *Domest Anim Endocrinol* 2002;23:265-75.
- Geisert RD, Renegar RH, Thatcher WW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. *Biol Reprod* 1982;27:925-39.
- Waclawik A. Novel insights into the mechanisms of pregnancy establishment: regulation of prostaglandin synthesis and signaling in the pig. *Reproduction* 2011;142:389-99.
- Seo H, Choi Y, Shim J, Yoo I, Ka H. Prostaglandin transporters *ABCC4* and *SLCO2A1* in the uterine endometrium and conceptus during pregnancy in pigs. *Biol Reprod* 2014;90:100.
- Schuster VL. Prostaglandin transport. *Prostaglandins Other Lipid Mediat* 2002;68-69:633-47.
- Kim M, Seo H, Choi Y, et al. Analysis of stage-specific gene expression profiles in the uterine endometrium during pregnancy in pigs. *PLoS One* 2015;10:e0143436.
- Samborski A, Graf A, Krebs S, et al. Transcriptome changes in the porcine endometrium during the preattachment phase. *Biol Reprod* 2013;89:134.
- Cole SP. Multidrug resistance protein 1 (MRP1, *ABCC1*), a "multi-tasking" ATP-binding cassette (ABC) transporter. *J Biol Chem* 2014;289:30880-8.
- Riches Z, Walia G, Berman JM, Wright TE, Collier AC. ATP-binding cassette proteins BCRP, MRP1 and P-gp expression and localization in the human umbilical cord. *Xenobiotica* 2016;46:548-56.
- Lovasz N, Ducza E, Gaspar R, Falkay G. Ontogeny of sulfonylurea-binding regulatory subunits of K(ATP) channels in the pregnant rat myometrium. *Reproduction* 2011;142:175-81.
- Kuo KL, Zhu H, McNamara PJ, Leggas M. Localization and functional characterization of the rat *Oatp4c1* transporter in an *in vitro* cell system and rat tissues. *PLoS One* 2012;7:e39641.
- Sebastian K, Detro-Dassen S, Rinis N, et al. Characterization of *SLCO5A1/OATP5A1*, a solute carrier transport protein with non-classical function. *PLoS One* 2013;8:e83257.
- Hagenbuch B, Meier PJ. Organic anion transporting polypeptides of the OATP/SLC21 family: phylogenetic classification as OATP/SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers Arch* 2004;447:653-65.
- Evseenko DA, Paxton JW, Keelan JA. Independent regulation of apical and basolateral drug transporter expression and function in placental trophoblasts by cytokines, steroids, and growth factors. *Drug Metab Dispos* 2007;35:595-601.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods* 2001;25:402-8.
- Olivier B, Walter W. A simplified *in situ* hybridization protocol using non-radioactively labeled probes to detect abundant and rare mRNAs on tissue sections. *Biochemica* 1998;1:10-6.
- Olbrich HG, Michaelis H, Vandeplassche G, et al. Ultrastructural calcium distribution and myocardial calcium content in human idiopathic dilated cardiomyopathy. *Cardiovasc Pathol* 1993;2:127-36.
- Aye IL, Paxton JW, Evseenko DA, Keelan JA. Expression, localisation and activity of ATP binding cassette (ABC) family of drug transporters in human amnion membranes. *Placenta* 2007;28:868-77.
- Lybaert P, Hoofd C, Guldner D, et al. Detection of K(ATP) channels subunits in human term placental explants and evaluation of their implication in human placental lactogen (hPL) and human chorionic gonadotropin (hCG) release. *Placenta* 2013;34:467-73.
- Blitek A, Waclawik A, Kaczmarek MM, et al. Expression of cyclooxygenase-1 and -2 in the porcine endometrium during the oestrous cycle and early pregnancy. *Reprod Domest Anim* 2006;41:251-7.
- Seo H, Choi Y, Shim J, Yoo I, Ka H. Comprehensive analysis of prostaglandin metabolic enzyme expression during pregnancy and the characterization of *AKR1B1* as a prostaglandin F synthase at the

- maternal-conceptus interface in pigs. *Biol Reprod* 2014;90:99.
26. Johnson GA, Bazer FW, Burghardt RC, et al. Conceptus-uterus interactions in pigs: endometrial gene expression in response to estrogens and interferons from conceptuses. *Soc Reprod Fertil Suppl* 2009;66:321-32.
27. Seo H, Kim M, Choi Y, Lee CK, Ka H. Analysis of lysophosphatidic acid (LPA) receptor and LPA-induced endometrial prostaglandin-endoperoxide synthase 2 expression in the porcine uterus. *Endocrinology* 2008;149:6166-75.
28. Seo H, Choi Y, Shim J, Choi Y, Ka H. Regulatory mechanism for expression of IL1B receptors in the uterine endometrium and effects of IL1B on prostaglandin synthetic enzymes during the implantation period in pigs. *Biol Reprod* 2012;87.
29. Geisert RD, Zavy MT, Wettemann RP, Biggers BG. Length of pseudopregnancy and pattern of uterine protein release as influenced by time and duration of oestrogen administration in the pig. *J Reprod Fertil* 1987;79:163-72.
30. Edgerton LA, Kaminski MA, Silvia WJ. Changes in uterine secretion of prostaglandin F2 alpha in response to oxytocin during the estrous cycle, early pregnancy, and estrogen-induced pseudopregnancy in swine. *Biol Reprod* 1996;55:657-62.