Porcine endometrial and conceptus tissue kallikrein 1, 4, 11, and 14 gene expression

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Abstract

Previous studies have suggested that the porcine endometrium may express several tissue kallikreins during the estrous cycle and early pregnancy. The present study investigated porcine endometrial and conceptus tissue kallikrein 1, 4, 11, and 14 mRNA expression during the estrous cycle and early pregnancy. Tissue kallikrein (*KLK*) gene expression was evaluated using quantitative RT-PCR and *in situ* hybridization. *KLK1* expression was similar across the estrous cycle and early pregnancy, and localized to the endometrial luminal (L) and glandular (G) epithelium. *KLK4* endometrial mRNA expression was greatest on days 0, 5, and 10 when compared with days 12, 15, and 17 of the estrous cycle and greater in cyclic compared with pregnant gilts. Expression of *KLK4* was more intense in the stroma and uterine epithelium from days 0 to 10 of the estrous cycle. Endometrial *KLK11* mRNA was not different between cyclic and pregnant gilts but the expression was greatest on days 10 and 12 compared with all other days evaluated. There was an increased intensity of *KLK11* gene expression in the stratum compactum on day 10 of the estrous cycle and early pregnancy. Endometrial *KLK14* mRNA expression was not detectable on days 5 and 10 but was expressed on days 0, 12, 15, and 17 of the estrous cycle and pregnancy. *KLK14* expression was localized in the uterine L and G epithelium, and stroma throughout the endometrium after day 10. Conceptus *KLK1* mRNA did not change from days 10 to 17 of gestation. However, conceptus *KLK4*, and 14 mRNA expression was greatest on day 10 with expression declining after day 14 of gestation. Expression of the various tissue kallikreins in the endometrium and conceptus during the estrous cycle and early pregnancy in the pig can serve in the activation of growth factors and tissue remodeling during the establishment of pregnancy.

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Introduction

Prior to initiating the epitheliochorial type of placental attachment to the uterine surface on day 13 of pregnancy, porcine conceptuses undergo rapid elongation from a 10 mm sphere to a long filamentous thread-like structure (Geisert *et al.* 1982, Stroband & Van de Lende 1990). Following trophoblast elongation, attachment to the uterine surface epithelium by the developing conceptuses stimulates a localized increase in transcapillary transport (Keys & King 1990) and uterine blood flow (Ford *et al.* 1982), which occurs concurrently with the establishment and maintenance of pregnancy (Geisert & Yelich 1997). The increase in uterine blood flow and many of the endometrial responses that occur during early porcine conceptus development resembles the acute-phase response induced during tissue inflammation (Salier *et al.* 1996, Geisert & Yelich 1997).

The tissue kallikrein family of serine proteases activate a wide range of substrates and growth factors (Chan *et al.*

1999), suggesting that kallikreins are involved in many integral processes of early embryonic development, such as regulation of uterine blood flow, angiogenesis, tissue invasion, and mitogenesis (Yousef & Diamandis 2003). Five tissue kallikrein genes have been detected in endometrium of mice and rats and are proposed to play a role in uterine physiological function during embryo implantation (Valdes *et al.* 1996, Corthorn *et al.* 1997, Chan *et al.* 1999). Detection of tissue kallikrein enzymatic activity in the porcine uterine lumen (Vonnahme *et al.* 1999), as well as the presence of the substrate for tissue kallikrein, low molecular weight (LMW) kininogen, in the porcine endometrium indicates an active kallikrein–kininogen–kinin system exists during early conceptus development and placental attachment (Vonnahme *et al.* 2004). Indeed, a pregnancy-specific increase in bradykinin release occurs during the period of trophoblast attachment to the uterine surface, indicating involvement of tissue kallikreins in establishment of pregnancy in the pig (Allen *et al.* 2002).

Recent studies have demonstrated that uterine tissue kallikreins are involved in the cleavage of insulin-like growth factor binding proteins in the uterine lumen on day 12 in cyclic and pregnant gilts (Geisert *et al.* 2001), suggesting that several different kallikreins may be present during early conceptus development. The tissue kallikrein family is encoded by a single gene cluster located at porcine chromosome region 6q12–21 (Fernando *et al.* 2006). Sequence analysis of the tissue kallikrein region revealed the presence of 13 tissue kallikrein genes in the porcine genome. It is possible that the uterine tissue kallikreins may be involved with extracellular matrix remodeling, degradation of insulin-like growth factor binding proteins, and bradykinin release during early conceptus development in the pig (Geisert *et al.* 1998, 2001). Previous isolation, sequencing, and tissue detection of the porcine tissue kallikrein genes indicated that four genes orthologous to human kallikrein (*KLK*) 1, 4, 11, and 14 are expressed abundantly in the endometrium (Fernando *et al.* 2006). Therefore, the present study characterized endometrial and conceptus *KLK1*, *KLK4*, *KLK11*, and *KLK14* mRNA expression during the porcine estrous cycle and early pregnancy.

Materials and Methods

Animals

Research was conducted in accordance with the Guiding Principles for Care and Use of Animals promoted by the Society for the Study of Reproduction and approved by the Oklahoma State Institutional Care and Use Committee. Cyclic, crossbred gilts of similar age (8–10 months) and weight (100–130 kg) were checked twice daily for the onset of estrus behavior (estrus onset = day 0 of estrous cycle) using intact boars. Gilts assigned for mating were bred naturally with fertile boars 12 h after the first detection of estrus onset and again 12 h later. Cyclic gilts (4 gilts/day) were hysterectomized on either day 0, 5, 10, 12, 15, or 17 of the estrous cycle, while pregnant gilts (4 gilts/day) were hysterectomized on either day 10, 12, 15, or 17 as described previously by Gries *et al.* (1989).

Following initial induction of anesthesia with 1.5 ml intramuscularly administered cocktail consisting of 2.5 ml Rompum (xylazine; 100 mg/ml; Phoenix Scientific Inc, St. Louis, MO, USA) and 2.5 ml Vetamine (ketamine HCl; 100 mg/ml; Mallinckrodt Veterinary, Mundelein, IL, USA) in 500 mg Telazol (tiletamine HCl and zolazepam HCl; Fort, Dodge, Syracuse, NE, USA), anesthesia was maintained with a closed circuit system of halothane (Halocarbon Laboratories, Riveredge, NJ, USA) and oxygen (1.0 l/min). The uterus was exposed via mid-ventral laparotomy and the uterus and ovaries were excised. Uterine luminal contents and conceptuses from bred females were flushed from the horn by infusing 20 ml PBS (pH 7.4) through the lumen and collecting flushings into a petri dish. Conceptuses were removed

from flushings, snap-frozen in liquid nitrogen and stored at -80°C . At hysterectomy, several sections (~ 0.5 cm) from the middle of each uterine horn were prepared for *in situ* hybridization by fixing in fresh 4% paraformaldehyde in PBS (pH 7.2) and embedding in Paraplast-Plus (Oxford Laboratory, St Louis, MO, USA). Endometrial tissue was removed from the antimesometrial side of the uterine horn, immediately snap-frozen in liquid nitrogen, and stored at -80°C until utilized for RNA extraction. To more closely characterize conceptus kallikrein mRNA expression across the early stages of embryo development, conceptuses were collected from an additional group of gilts (3 gilts/day) hysterectomized on days 11, 13, 14, 16, and 17 of gestation.

RNA extraction from endometrial tissue

Following addition of 4 ml TRIzol (Invitrogen) to approximately 1 g endometrial tissue, samples were homogenized using a Virtishear homogenizer (Virtis Company, Inc., Gardiner, NY, USA). Samples were incubated at room temperature for 5 min and 1 ml chloroform was added to the sample, incubated at room temperature for 3 min and then centrifuged at 4°C for 30 min at 5000 g. The aqueous phase was transferred into a fresh tube, 2.5 ml isopropyl alcohol added, placed in a -80°C freezer for 20 min and centrifuged at 4°C for 30 min at 22 500 g. Supernatant was discarded and the pellet was washed with 3 ml of 75% ethanol and then air-dried for 5 min. Total RNA was resuspended in 500 μl diethyl pyrocarbonate (DEPC)-treated water and further purified by phenol:chloroform:isoamyl alcohol extraction followed by ethanol precipitation. Samples were treated with DNase I (Invitrogen) according to the manufacturer's protocol to eliminate possible DNA contamination. Total RNA was quantified with a spectrophotometer at an absorbance of 260 nm and purity was verified based on the ratio of 260:280. Integrity of the extracted RNA was further evaluated by electrophoresis, using a fraction of the RNA extract on a 1.0% agarose denaturation gel containing 1.68% formaldehyde.

RNA extraction from conceptus tissue (RNAWIZ)

A tenfold volume of RNAWIZ (Ambion, Austin, TX, USA) was added to 1 volume conceptus tissue and the mixture vortexed until the tissue was completely homogenized. The solution was incubated at room temperature for 5 min, 0.2 $\times$ starting volume of chloroform was added to the homogenate, vortexed for ~ 20 s and then incubated at room temperature for 10 min. After incubation, sample was centrifuged at 4°C for 30 min at 16 000 g, aqueous phase was transferred into a fresh tube, and 0.5 $\times$ starting volume of RNase-free water was added and mixed well. Isopropanol (1 $\times$) was immediately added and sample placed in a -80°C ultralow freezer. Following a 30-min freezing period, the sample was then

centrifuged at 4 °C for 15 min at 16 000 *g*, supernatant discarded and pellet washed with 3 ml of 75% ethanol. The resulting pellet was resuspended in 50 µl DEPC-treated water and samples treated with DNase I (Invitrogen) according to the manufacture's protocol.

Quantitative real-time PCR

Quantitative analysis of porcine endometrial and conceptus *KLK1*, *KLK4*, *KLK11*, and *KLK14* mRNA were conducted using quantitative real-time reverse transcriptase-PCR (qRT-PCR) as previously described (Hettinger *et al.* 2001). The transcripts for *KLK1*, *KLK4*, and *KLK14* were evaluated using dual labeled probes containing 6-Fam (5' reporter dye), and TAMRA (3' quenching dye). Primers and probes for *KLK1*, *KLK4*, and *KLK14* (Table 1) were developed from porcine-specific sequences (GenBank AC149292). Quantification of *KLK11* (primers presented in Table 1) was evaluated using SYBR green reporter assay kit available from Qiagen. All primers and probes were developed using Primer3 (Rozen & Skaletsky 2000) and software available at IDT (Integrated DNA Technologies; <http://scitools.idtdna.com/Primerquest/>) and were designed to cross an intron to prevent genomic DNA amplification. All PCR products were sequenced to confirm identity of the *KLK*. For qRT-PCR of *KLK1*, 4 and 14, a total reaction volume of 25 µl contained 400 nM forward primer, 400 nM reverse primer, 200 nM dual labeled fluorescent probe, 1× Master mix (Qiagen), 12.5 U RT, and 100 ng total RNA. Thermal cycling conditions were 50 °C for 30 min, 95 °C for 10 min, followed by 45 additional cycles of 95 °C for 15 s, and 60 °C for 1 min. Thermal cycling conditions used for SYBR green detection of *KLK11* were 50 °C for 30 min, 95 °C for 10 min, followed by 45 additional cycles of 95 °C for 15 s and 61 °C for 30 s, and 72 °C for 1 min. Ribosomal 18S RNA (18S RNA Control Kit; Eurogentec, Philadelphia, PA, USA) was assayed according to the manufacturer's protocol for each sample for normalization. The qRT-PCR reactions were performed in an ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA).

Template amplification was quantified by determining the threshold cycle (C_T) based on the fluorescence detected within the geometric region of the semi-log plot. Theoretically, in the geometric region, one cycle is equivalent to the doubling of PCR target template. A tenfold dilution series starting from 1 µg to 100 pg was made for each assay to determine the actual PCR efficiency and the dynamic range, which resulted in correlation coefficients of 0.982, 0.965, 0.996, and 0.996 for *KLK1*, *KLK4*, *KLK11*, and *KLK14* respectively. Using the comparative C_T method (Hettinger *et al.* 2001), relative quantification of mRNA abundance was determined for the endometrial samples. Sample expressions of *KLK1*, *KLK4*, *KLK11*, and *KLK14* mRNA were normalized with ribosomal 18S expression to adjust for sample loading. Relative units of target mRNA expression were calculated by arbitrarily setting the lowest sample measurement (C_T) as one relative unit, which was utilized as the baseline standard to compare mRNA abundance for all other samples. Relative mRNA abundance was calculated using the PCR efficiencies for *KLK1*, *KLK4*, *KLK11*, and *KLK14* in the formula 2^{-C_T} .

In situ hybridization

In situ hybridizations were performed using digoxigenin (DIG)-labeled cRNA probes using the protocol described by Adelson *et al.* (2004). PCR primers were designed with the forward primer having the T7 RNA polymerase site incorporated to the 5' end of the primer, whereas the T3 RNA polymerase site was incorporated to the 5' end of the reverse primer (Table 2). *KLK1*, *KLK4*, *KLK11*, and *KLK14* were amplified by RT-PCR as described by Fernando *et al.* (2006). A DIG RNA Labeling kit (Roche Diagnostics) was used according to the manufacture's protocols to synthesize the sense and antisense RNA from the amplified PCR product. The RNA probes synthesized were quantified and an equal amount of each probe was used for hybridization.

Paraffin-embedded tissue was serially sectioned (6 µm) and mounted on positively charged glass microscope slides. The slides were deparaffinized in

Table 1 Porcine PCR primer and probe sequences used for quantitative RT-PCR.

Gene		Forward primer/reverse primer/probe
<i>KLK1</i>	Forward	5'-GGACTACAGCCACGACCTCATGCTGC-3'
	Reverse	5'-GTCGGGGAATTCGAAGTCGTGG-3'
	Probe	5'-TGGAGCTGCCACCCAGGAACCCGAA-3'
<i>KLK4</i>	Forward	5'-ATCCGCAATGGGTGCTGTCA-3'
	Reverse	5'-GGGTTCTTGTTCGGGCTCAAGATT-3'
	Probe	5'-ATTCCTACACCATCGGGCTGGGTC-3'
<i>KLK11</i> ^a	Forward	5'-CGTCCAGCCCCAGTTG-3'
	Reverse	5'-CCATGGTGTCCGTGATGTTG-3'
<i>KLK14</i>	Forward	5'-ATGTTCTCTGCTGACAGCACT-3'
	Reverse	5'-CAGAGGAAACGATGCCAGGACC-3'
	Probe	5'-GCCACGGCACAGAGCCAGGGCAATGAGAACAAGATAAT-3'

^aExpression of mRNA determined with SYBR green.

Table 2 Porcine PCR primers used for cRNA synthesis for *in situ* hybridization experiments.

Gene	Forward primer/reverse primer
<i>KLK1</i>	5'-TAATACGACTCACTATAGGGGACTACAGCCACGACCTCATGCTGC-3' 5'-AATTAACCCCTCACTAAAGGCACCCATACAGGTGTCTTGCCACCT-3'
<i>KLK4</i>	5'-TAATACGACTCACTATAGGGTGGCTTCTGAGGAGGTCTG-3' 5'-AATTAACCCCTCACTAAAGGGCCTGGATGGTCTGCTGTAT-3'
<i>KLK11</i>	5'-TAATACGACTCACTATAGGTGTCGGGGGAGAGACTAGGA-3' 5'-AATTAACCCCTCACTAAAGGCGGCAGTGACACACTGTGAA-3'
<i>KLK14</i>	5'-TAATACGACTCACTATAGGATGTTCTCTGCTGACAGCACTTCA-3' 5'-AATTAACCCCTCACTAAAGGGTGGGTCCGGGAGTTGTACT-3'

xylene and serially rehydrated in 100–50% ethanol and PBS. The rehydrated slides were digested with proteinase K (10 µg/ml; Invitrogen) for 10 min at 60 °C. The proteinase K-treated slides were then acetylated at room temperature for 15 min to minimize background signal. Upon acetylation, the slides were prehybridized in 50% formamide (Sigma-Aldrich), 2× SSC, and salmon sperm DNA (50 µg/ml) for 2 h at 53 °C. The sections were then hybridized with DIG-labeled sense or antisense probes (5 ng/µl) at 53 °C for 16 h in the presence of 50% formamide (Sigma-Aldrich), 4× SSC, 50 µg/ml yeast tRNA, 1× Denhardt's solution, and 100 mg dextran sulfate solution. Following hybridization, unbound cRNA probes were removed by washing with 2× SSC/50% formamide at 55 °C for 15 min, digestion with 50 µg/ml RNase A (Invitrogen) at 55 °C for 30 min and washing in 1× SSC/50% formamide for 10 min at 55 °C. The sections were then incubated for 2 h in 1% DIG-blocking reagent (Roche Diagnostics) and was treated with anti-DIG conjugated with alkaline phosphatase for 60 min and was visualized using the DIG Nucleic Acid detection Kit (Roche Diagnostics). The endogenous alkaline phosphatase activity was blocked by the addition of 5 mM levamisole chromogenic reagent solution. The color development was stopped by immersing the slides in TE buffer.

Statistical analysis

Data were analyzed by the least square ANOVA using the PROC MIXED procedure of SAS (1988). The statistical model used to analyze endometrial gene expression (ΔC_T value) included effects of day, reproductive status (cyclic and pregnant), and the day×reproductive status interaction. The fixed effect of day was tested for conceptus *KLK* mRNA expression. Results are presented as least square means $\pm$ S.E.M.

Results

Endometrial and conceptus *KLK1*, *KLK4*, *KLK11*, and *KLK14* expressions

Endometrial and conceptus *KLK1*, *KLK4*, *KLK11*, and *KLK14* mRNA expressions were analyzed using quantitative real-time PCR and *in situ* hybridization.

Endometrial *KLK1* mRNA expression was detected across all days of the estrous cycle and early pregnancy (Fig. 1), but endometrial *KLK1* mRNA expression was not effected by day or status ($P < 0.10$). *KLK1* expression was localized in the uterine luminal (L) and glandular (G) epithelium (Fig. 2). Conceptus *KLK1* mRNA expression was similar across all days of gestation evaluated (Fig. 4).

Quantitative RT-PCR analysis of endometrial *KLK4* mRNA expression (Fig. 1) revealed a significant day ($P < 0.05$) and status ($P < 0.01$) effect on mRNA expression. Endometrial *KLK4* mRNA expression was greatest during the early days of the estrous cycle (days 0–10) compared with the later stages (days 12–17) of the estrous cycle and early pregnancy (Fig. 1). Endometrial *KLK4* expression was detected in the uterine epithelium and stroma (Fig. 3). The intensity of *KLK4* mRNA expression was greatest on days 5–10 of the estrous cycle and early pregnancy. Stromal *KLK4* expression was particularly pronounced during the early stages of the estrous cycle with intensity declining after day 10. However, *KLK4* expression continued to be evident in the epithelium and the stratum compactum to day 15 of the estrous cycle and pregnancy. Expression of porcine *KLK4* mRNA by the developing conceptuses declined following day 14 of gestation as the lowest expression was detected on day 17 of gestation (Fig. 4).

Endometrial *KLK11* mRNA expression was effected by day ($P < 0.05$) but not status (Fig. 1). *KLK11* endometrial mRNA was lowest on days 0 and 5 of the estrous cycle. Expression of *KLK11* increased 14- and 8-fold on days 10 and 12 of the estrous cycle and pregnancy (Fig. 1). The increase in endometrial *KLK11* mRNA was followed by a decline in expression on days 15 and 17. Endometrial *KLK11* mRNA expression was detected in the uterine epithelium and stroma (Fig. 3). The intensity of *KLK11* expression in the stroma (S) was increased on days 10 and 12 with a clear decline observed on day 17 of the estrous cycle and pregnancy. Expression of porcine *KLK11* mRNA by the developing conceptuses was approximately four- to five-fold greater ($P < 0.05$) prior to and during the period of trophoblast elongation on day 12. Conceptus *KLK11* expression significantly ($P < 0.05$) declined after day 16 of gestation (Fig. 4).

Expression of *KLK14* mRNA was not detected in days 5 and 10 cyclic or day 10 pregnant endometrium after 40

Figure 1 Relative units (mean $\pm$ S.E.M.) of mRNA expressions for endometrial *KLK1*, *KLK4*, *KLK11*, and *KLK14* during the porcine estrous cycle (white bar) and early pregnancy (black bar). Abundance of mRNA was calculated from the real-time PCR analysis as described in Materials and Methods. Bars with different superscripts represent a statistical difference in day ($P < 0.001$).

cycles of qRT-PCR. A day effect ($P < 0.01$) was detected for endometrial *KLK14* mRNA as endometrial *KLK14* mRNA expression was detected on days 0, 12, 15, and 17 (Fig. 1). Endometrial *KLK14* mRNA expression was not different between cyclic and pregnant gilts from days 10 to 17. Expression of *KLK14* was not evident in the endometrium of gilts collected on days 5 and 10 of the estrous cycle and day 10 of pregnancy (Fig. 3). Endometrial *KLK14* mRNA expression was detected in the uterine L and G epithelium and S stratum compactum from days 12 to 17 of the estrous cycle and pregnancy. Quantitative RT-PCR analysis of conceptus *KLK14* mRNA expression revealed a day effect

($P < 0.05$) as expression of *KLK14* mRNA declined fourfold after day 16 gestation (Fig. 4).

Discussion

The role of the various tissue kallikreins in altering extracellular matrix suggests that the serine proteases are involved with uterine implantation and early conceptus development in the pig. Tissue kallikreins are proposed to be involved with the invasive implantation and conceptus development in the rat (Corthorn & Valdes 1994, Valdes *et al.* 1996) and mouse (Chan *et al.* 1999). The tissue kallikreins belong to a large gene family in which 25 members (14 genes that encode for serine proteases and 11 are pseudogenes) are present in the mouse, 13 in the rat, and the human kallikrein family consists of 15 genes (Yousef & Diamandis 2003, Diamandis *et al.* 2004). The 13 porcine tissue kallikrein genes are clustered on chromosome 6 (Ferando *et al.* 2006) and the pig lacks orthologs to human *KLK2* and *KLK3* as occurs in rodents (Yousef & Diamandis 2003). Tissue kallikreins are known to regulate the activation of many growth factors and extracellular proteases in a variety of tissues (Clements 1997). Expression for a number of the tissue kallikreins is regulated by androgens and estrogens, which contributes to tissue kallikrein expression in reproductive organs including the ovary, uterus, placenta, and mammary gland (Yousef & Diamandis 2001). Although tissue kallikreins have long been utilized as biomarkers for human prostatic, ovarian, and breast cancer (Yousef & Diamandis 2003), the specific roles for a number of the various kallikreins is largely unknown. Chan *et al.* (1999) detected expressions of *Klk1*, *Klk3*, *Klk9*, and *Klk21* in

Figure 2 *In situ* hybridization analysis of *KLK1* mRNA expression in porcine endometrium during day 15 of the estrous cycle and day 17 of pregnancy. A representative section of day 15 cyclic and day 17 pregnant endometrium was hybridized with DIG-labeled cRNA probe (sense) to serve as a negative control (L, luminal epithelium; G, glandular epithelium; and S, stroma). Magnification $\times 260$.

KLK4 **KLK11** **KLK14**

Figure 3 *In situ* hybridization analysis of *KLK4*, *KLK11*, and *KLK14* mRNA expression in porcine endometrium during the estrous cycle (C) and pregnancy (P). Protected transcripts in endometrium from days 5, 10, 12, 15, and 17 of the estrous cycle and days 10, 12, 15, and 17 of pregnancy were detected using DIG-labeled cRNA probes and were visualized using anti DIG alkaline phosphatase conjugate and a NBT/BCIP substrate. A DIG-labeled sense cRNA probe (Sense) was used as a negative control (L, luminal epithelium; G, glandular epithelium; and S, stroma). Magnification $\times 260$.

early mouse embryos and *Klk1* and *Klk21* mRNA expressions in the endometrium and decidua during pregnancy. Porcine endometrial expressions of *KLK1*, *KLK4*, *KLK6*, *KLK9*, *KLK10*, *KLK11*, *KLK14*, and *KLK15* were detected by RT-PCR (Fernando *et al.* 2006). However, endometrial expressions of *KLK4*, *KLK9*, *KLK11*, and *KLK14* were particularly abundant in comparison with other tissues of body. The results of our study indicated that there is differential expression pattern for the four tissue kallikrein genes in the porcine endometrium during the estrous cycle and early pregnancy.

Functionally, *KLK1* is considered the major tissue kallikrein serine protease, which liberates bradykinin from LMW-kininogen (Clements 1997). In the rat, the greatest increase in tissue kallikrein is seen in the uterine glandular epithelium (Valdes *et al.* 1996) and gene expression coincides with the period of trophoblast invasiveness (Chan *et al.* 1999). Endometrial levels of *KLK1* are also increased during the proliferative phase of the human menstrual cycle, which suggests estrogen regulation of the *KLK1* gene (Clements *et al.* 1994). We have previously detected endometrial *KLK1* mRNA

Figure 4 Relative units (mean $\pm$ S.E.M.) of mRNA expressions for conceptus *KLK1*, *KLK4*, *KLK11*, and *KLK14* during early pregnancy. Abundance of mRNA was calculated from the real-time PCR analysis as described in Materials and Methods. Bars with different super-scripts represent a statistical difference in day ($P < 0.001$).

and protein expression during early pregnancy in the pig (Vonnahme *et al.* 1999). *KLK1* enzymatic activity, which LMW kininogen is one of its specific substrates to release bradykinin, increases after day 12 of the porcine estrous cycle and early pregnancy (Allen *et al.* 2002). Although there is an increase in bradykinin release during early pregnancy, endometrial and conceptus expression of *KLK1* does not change across the days of the estrous cycle and early pregnancy (Vonnahme *et al.* 1999, present study). It is possible that conceptus induction of bradykinin release within the uterine lumen may occur through the cleavage of plasma prekallikrein by Factor XII (Vonnahme *et al.* 2004) rather *KLK1*. However, gene expression of endometrial plasma kallikrein is very low and not detectable in the conceptus. Detection of *KLK1* expression in the uterine L and G epithelium suggests that *KLK1* secreted into the uterine lumen could be activated through cleavage by conceptus proteases during attachment to the uterine surface. However, the pregnancy-specific increase of bradykinin release in the uterine lumen needs to be determined.

The present study clearly indicates there is a differential expression of the three other endometrial tissue kallikreins across the days of the estrous cycle and early pregnancy in the pig. Although *KLK4* is a secreted serine protease, also known as prostate and/or enamel matrix serine protease, it has a unique structure and function compared with other kallikrein family members (Korkmaz *et al.* 2001). This serine protease was first identified from the epithelial tissue of developing teeth. In the porcine dental enamel, *KLK4* is expressed during early transition and maturation stages of enamel formation where *KLK4* participates in the degradation

of enamel proteins such as amelogenin (Ryu *et al.* 2002). Expression of *KLK4* is up-regulated by estrogen and progesterone in the human endometrial cancer cell line, KLE (Myers & Clements 2001). The ability of *KLK4* to cleave a broad range of peptides and its localization in the porcine uterine epithelium and strong expression in the stroma on days 0, 5, and 10 estrous cycle and day 10 of pregnancy suggests that, *KLK4* may play a role in cellular growth and extracellular matrix remodeling before conceptus attachment to the uterine surface.

Conceptus *KLK4* expression is greatest on days 10–13 compared with days 15–17 of conceptus development. This time period is consistent with the time the porcine conceptus starts cell proliferation and placental membrane formation, which suggests that *KLK4* may be one of the factors assisting with conceptus extracellular matrix degradation, cellular remodeling, and attachment to the uterine luminal epithelium during embryonic development in the pig.

Kallikrein 11, previously referred to as hippostasin from its previous isolation from the human hippocampus is another of the tissue kallikreins that is highly expressed in the prostate epithelium and is proposed to be under steroid regulation (Stavropoulou *et al.* 2005). Since *KLK11* is proposed to function as a trypsin-like protease, it may function in the plasticity of the extracellular matrix and/or cleavage of proteins (Mitsui *et al.* 2000). The pattern of porcine endometrial *KLK11* mRNA expression was different from the other three tissue kallikreins investigated in our study. The increase of endometrial *KLK11* mRNA in the uterine epithelium and especially the stroma on days 10 and 12 is temporally associated with the time of rapid conceptus development

and establishment of pregnancy in the pig. However, the increase in endometrial *KLK11* mRNA is independent of the presence of conceptuses. Conceptus *KLK11* gene expression decreased following trophoblast elongation (day 12) and initiation of uterine attachment. Endometrial *KLK11* mRNA also decreased following day 12 indicating a very precise regulation of *KLK11* expression during the establishment of pregnancy in the pig.

Endometrial mRNA expression of *KLK14* is the most dynamic of any of the tissue kallikreins we evaluated in the porcine uterus. Endometrial *KLK14* mRNA expression was not detected on days 5 and 10 of the estrous cycle and early pregnancy. Although previous studies of *KLK14* have indicated that the expression is regulated through androgens and estrogens (Borgono *et al.* 2003, Yousef *et al.* 2003), our data suggest that *KLK14* mRNA expression may be negatively regulated by the presence of progesterone receptor (PR) in the uterine epithelium. Absence of endometrial *KLK14* mRNA expression on days 5 and 10 estrous cycle and early pregnancy in the pig is temporally associated with the presence and activation of uterine epithelial PR. During the porcine estrous cycle and early pregnancy, progesterone stimulates the myometrium, stroma, and uterine epithelium through its receptor. However, following approximately 8–10 days of progesterone stimulation there is a cell-specific loss of PR expression from porcine uterine L and G epithelium on day 12 of the estrous cycle or pregnancy (Geisert *et al.* 1994). The strong expression of *KLK14* in the L and G epithelium of the uterus after day 10 of the estrous cycle and early pregnancy suggests that progesterone may inhibit *KLK14* mRNA expression in uterine epithelium through progesterone activation of PR in the early stages of the estrous cycle. Human *KLK14* has trypsin- and chymotrypsin-like activity (Felber *et al.* 2005) for which components of the extracellular matrix, laminin α -5 and collagen IV, are potential substrates of *KLK14*. It is possible that the increase in *KLK14* mRNA could function in uterine remodeling during establishment of pregnancy in the pig. The spatiotemporal expressions of *KLK11* and *KLK14* were also consistent with the release of many endometrial cytokines (Geisert & Yelich 1997) and cleavage of the IGFBPs present in the uterine lumen of the pig (Geisert *et al.* 2001). The increase in endometrial *KLK11* and *KLK14* expression during a critical stage of porcine conceptus development suggests that these tissue kallikreins could serve to activate many factors involved with the establishment of pregnancy the pig.

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