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Klotho : Expression and Regulation at the Maternal-Conceptus Interface in Pigs

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ABSTRACT

Klotho (KL) is a single transmembrane protein composed of KL1 and KL2 repeats possessing β-glucuronidase activity and maintains calcium homeostasis in physiological state. It has been implicated in pigs that calcium is important for the establishment and maintenance of pregnancy, and our previous study has shown that transient receptor potential vanilloid type 6 (TRPV6), a calcium ion transporter, is predominantly expressed in the uterine endometrium during pregnancy in pigs. However, expression and function of KL in the uterine endometrium has not been determined in pigs. Thus, the present study determined expression and regulation of KL in the uterine endometrium during the estrous cycle and pregnancy in pigs. Real-time RT-PCR analysis showed that levels of *KL* mRNA decreased between Days 12 to 15 of the estrous cycle, and its expression showed a biphasic manner during pregnancy. *KL* mRNA was expressed in conceptuses and in chorioallantoic tissues during pregnancy. Explant culture study showed that expression levels of *KL* were not affected by treatment of steroid hormones or interleukin-1beta during the implantation period. Furthermore, levels of *KL* mRNA in the uterine endometrium from gilts carrying somatic cell nuclear transfer (SCNT)-derived embryos were significantly lower than those from gilts carrying natural mating-derived embryos on Day 12 of pregnancy. These results exhibited that *KL* was expressed at the maternal-conceptus interface in a pregnancy status- and stage-specific manner, and its expression was affected by SCNT procedure, suggesting that KL may play an important role in the establishment and maintenance of pregnancy in pigs.

(Key words : pig, uterus, KL, endometrium, calcium)

INTRODUCTION

Calcium ion (Ca^{2+}) is an important component in various physiological events in the uterus including embryo implantation, placental differentiation, and fetal bone mineralization processes during pregnancy (Baczyk *et al.*, 2011). During the implantation period in pigs, conceptuses (embryos/fetuses and associated extraembryonic membranes) undergo a dramatic morphological change and synthesize estrogen, the signal for maternal recognition of pregnancy, to protect the corpus luteum from luteolysis (Bazer and Thatcher, 1977). Conceptus-derived estrogen stimulates significant increases in Ca^{2+} levels in the uterine lumen between Days 11 and 12 of pregnancy (Geisert *et al.*, 1982). After the embryo implantation process, intracellular Ca^{2+} signaling modulates proliferation, differentiation, and migration of trophoblatic cells and contributes the proper placental function (Hellman *et al.*, 1993; Gan *et al.*, 2008). Considerable amounts of Ca^{2+} cross the trophoblastic layer during a successful pregnancy (Lafond *et al.*, 2001), and the Ca^{2+} transport from mother to the developing fetus is required to sustain adequate mineralization of fetal skeleton (Salle *et al.*, 1987). However, molecular and cellular mechanisms controlling changes of Ca^{2+} levels in the porcine uterus are not fully understood.

Ca^{2+} is absorbed by para- and transcellular transport pathways from diet and urine in the intestine and kidney, respectively (Hoenderop, 2002). A continuous epithelial layer regulates the exchange of Ca^{2+} flowing passively by the paracellular transport pathway. Tight junction at the intercellular region formulates the barrier to transport Ca^{2+} and small solutes through this path-

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way. The transcellular Ca^{2+} transport pathway is comprised of three steps: 1) extracellular Ca^{2+} uptake into cytosol by transient receptor potential vanilloid type 5 (TRPV5) and 6 (TRPV6); 2) intracellular Ca^{2+} transport by Ca^{2+} binding protein d9k (S100G); and 3) Ca^{2+} extrusion by plasma Ca^{2+} ATPases (ATP2Bs), Na^{+}/Ca^{2+} exchangers (NCXs) and K^{+}-dependent Na^{+}/Ca^{2+} exchangers (NCKXs) (Hoenderop, 2002; 2005). Our previous study showed expression of these calcium regulatory molecules in the uterine endometrium during the estrous cycle and pregnancy (Choi *et al.*, 2009; 2012; 2014), and endometrial expression of TRPV6 and S100G is increased by conceptus-derived estrogen during the implantation period in pigs (Choi *et al.*, 2009; 2012). Especially, expression levels of TRPV6 transcript and protein in the uterine endometrium are maintained throughout pregnancy, and they are exclusively localized to luminal epithelial cells during pregnancy.

Klotho (KL) is a type I single-pass transmembrane protein composed of a short intracellular domain and an extracellular domain including two repeats (KL1 and KL2) with weak homology to β-glycosidase family that hydrolyze β-glucosidic linkage (Kuro-o *et al.*, 1997; Mian, 1998). KL protein is primarily localized to plasma membrane, however, the extracellular domain of KL is cleaved by membrane-anchored protease, ADAM10 and ADAM17 (Chen *et al.*, 2007). For this reason, the two forms of KL protein, membrane-bound and secreted forms, are present and exhibit differential functions in the physiological state. Membrane-bound KL functions as a canonical co-receptor with fibroblast growth factor (FGF) receptor (FGFR) for FGF23 in kidney (Kuro-o, 2006; Urakawa *et al.*, 2006). Because FGF23 binds to its receptor (FGFR) with low affinity, a FGF23-FGFR complex is not able to stimulate its downstream signaling pathway without membrane-bound KL (Yu *et al.*, 2005). Membrane-bound KL is an essential mediator required for FGF23-induced intracellular signaling pathway that regulates 1,25-dihydroxyvitamin D_3($1,25(OH)_2D_3$) and phosphate homeostasis in kidney (Shimada *et al.*, 2004; Liu and Quarles, 2007; Segawa *et al.*, 2007). Secreted KL (sKL) acts as a soluble enzyme possessing β-glucuronidase activity that regulates activities of TRPV5 and TRPV6 channels, glycosylated in a conserved N-glycosylation site of the extracellular loop between the first and second transmembrane domains (Huang, 2004), by hydrolyzing the glycosylation moiety in the extracellular domain of these channels (Chang *et al.*, 2005; Cha *et al.*, 2008; Lu *et al.*, 2008). The sialic acid moiety at the terminals in N-linked glycan is removed from TRPV5 and TRPV6 channels by sKL (Cha *et al.*, 2008) and soluble galectin-1 that binds to exposed galactose and N-acetyl lactosamine of the remained N-linked glycan induces the assembly of channels, resulting in accumulation of TRPV5 and TRPV6 on plasma membrane (Huang, 2004). It leads us to hypothesize that KL is expressed in the uterine endometrium and contributes accumulation of TRPV6 protein on the cell surface of uterine epithelial cells during pregnancy in pigs.

Recently, somatic cell nuclear transfer (SCNT) technique draws attention because it is able to generate cloned animals for both basic and applied researches, but the efficiency of generating viable cloned animals by SCNT technique is extremely low. This low efficiency is caused by several reasons, including abnormal extra-embryonic tissue formation (Chae *et al.*, 2006; Jouneau *et al.*, 2006; Kim *et al.*, 2009) and the inappropriate uterine responsiveness to conceptuses (Kim *et al.*, 2005; Ka *et al.*, 2008). Identification of aberrant gene expression in the uterus with SCNT-derived conceptuses is required to improve the efficiency of obtaining viable cloned animals. Our recent studies have shown that various uterine endometrial genes, involved in calcium regulation, immune response, and lysophosphatidic acid (LPA)-mediated cell signaling pathway, are aberrantly expressed in the uterine endometrium of gilts with SCNT-derived conceptuses on Day 12 of pregnancy (Choi et al,. 2012; Kim *et al.*, 2012; Seo *et al.*, 2013). We hypothesized that the SCNT procedure may affect expression of KL in the uterine endometrium, and, turn in, induce abnormal calcium regulatory mechanisms during the implantation period.

Therefore, the aim of present study was to demonstrate the followings: 1) expression and localization of KL in the uterine endometrium during the estrous cycle and pregnancy; 2) expression of KL in conceptuses during early pregnancy; 3) effects of steroid hormones and interleukin-1beta (IL1B) on KL expression in the uterine endometrium; and 4) effect of SCNT procedure on expression of KL in the uterine endometrium on Day 12 of pregnancy in pigs.

MATERIALS AND METHODS

1. Animals, Embryo Transfer and Tissue Collection

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. The reproductive tracts of gilts were obtained immediately after slaughter on either Day 12 (n=6) or Day 15 (n=4) of the estrous cycle and either Day 12 (n=6), Day 15 (n=6), Day 30 (n=4), Day 60 (n=3), Day 90 (n=3) or Day 114 (n=4) of pregnancy. Pregnancy was confirmed by the presence of apparently normal filamentous conceptuses in uterine flushings on Days 12 and 15 and the presence of embryos and placenta on the later days of pregnancy. Chorioallantoic tissues were obtained from Day 30 (n=4), Day 60 (n=3), Day 90 (n=3) and Day 114 (n=4) of pregnancy. Uterine flushings were obtained by introducing and recovering 50 ml phosphate buffered saline (PBS) (pH 7.4) after hysterectomy (25 ml/uterine horn). Endometrium, dissected free of myometrium, was colleceted from the middle portion of each uterine horn, snap-frozen in liquid nitrogen, and stored at −80℃ for RNA extraction.

Oocyte collection, *in vitro* maturation, somatic cell nuclear transfer (SCNT) procedures and endometrial tissue sampling from gilts carrying cloned embryos on Day 12 of pregnancy were done as described previously (Ka *et al.*, 2008; Kim *et al.*, 2009). On Day 12 of pregnancy, uterine endometrial tissues were obtained from four gilts that carried SCNT-derived conceptuses and three gilts with conceptuses resulting from natural mating.

2. Explant Culture

Endometrium was dissected from the myometrium and placed into warm phenol red-free DMEM/F-12 culture medium (DMEM/F-12; Sigma, St. Louis, MO) containing penicillin G (100 IU/ml; Sigma) and streptomycin (0.1 mg/ml; Sigma) as described previously (Ka *et al.*, 2001), with some modifications. The endometrium was minced with scalpel blades into small pieces (2～3 mm^3), and aliquots of 500 mg were placed into T25 flasks with serum-free modified DMEM/F-12 containing 10 μg/ml insulin (catalog no. I5500; Sigma), 10 ng/ml transferrin (catalog no. T1428; Sigma), and 10 ng/ml hydrocortisone (catalog no. H0396; Sigma). Endometrial explants were cultured immediately after mincing in the presence of ethanol (control), estradiol-17β (E_2; 50 ng/ml; catalog no. E8875; Sigma), progesterone (P_4; 3 ng/ml; catalog no. P0130; Sigma), E_2+P_4, E_2+P_4+ICI182,780 (ICI; an estrogen receptor antagonist; 100 ng/ml; Tocris, Ballwin, MO) or E_2+P_4+ RU486 (a progesterone receptor antagonist; 30 ng/ml; catalog no. M8046; Sigma), for 24 h with rocking in an atmosphere of 5% carbon dioxide in air at 37℃. To determine the effects of cytokines on *KL* expression, explant tissues were treated with 0, 1, 10 or 100 ng/ml IL1B (catalog no. I9401; Sigma) in the presence of both E_2 (50 ng/ml) and P_4 (3 ng/ml) at 37℃ for 24 h. Explant tissues were then harvested and total RNA was extracted for real-time RT-PCR analysis of *KL* mRNA levels. These experiments were conducted using endometrium from three individual gilts. Treatments were performed in triplicate on tissues obtained from each gilt.

3. Total RNA Extraction and Cloning of Porcine Calcium Regulatory Genes

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

Two micrograms of total RNA were treated with DNase I (Promega, Madison, WI) and reverse transcribed using Super-Script II Reverse Transcriptase (Invitrogen) to obtain cDNA. The cDNA templates were then diluted 1:4 with sterile water and amplified by PCR using Taq polymerase (Takara Bio, Shiga, Japan) and specific primers (forward, 5'-CGG GGT TCC TTT GAC TTT TTG G-3'; reverse, 5'-TGG TTT TGG CTC AAA CTG ATT TGC-3') based on mRNA sequences of porcine *KL* (Ensembl ID #: ENSSSCT00000010244). PCR conditions were 40 cycles at 94℃ for 30 sec, 60℃ for 30 sec and 72℃ for 1 min. PCR products with the expected size of 426 bp were separated on 2% agarose gels and visualized by ethidium bromide staining. The identity of each amplified PCR product was verified by sequence analysis after cloning in to the pCRII vector (Invitrogen).

4. Real-time Quantitative RT-PCR

To analyze levels of *KL* mRNAs in the uterine endometrium, real-time RT-PCR was performed using the Applied Biosystems StepOnePlus System (Applied Biosystems, Foster City, CA) using the SYBR Green method. Complementary DNAs were synthesized from 3 μg of total RNA isolated from different uterine endometrial tissues, and newly synthesized cDNAs (total volume of 21 μl) were used for PCR. To maximize efficiency,

specific primers based on porcine *KL* (Ensembl ID #: ENSSSCT00000010244, forward, 5'-CAA TTT CCT CCT CCC TTA TTT CAC G-3'; reverse, 5'-GCG AGG TAG TCG TTG TAT TTT GTC G-3') and porcine ribosomal protein L7 (*RPL7*) (GenBank accession # NM_0011132176, Forward: 5'-AAG CCA AGC ACT ATC ACA AGG AAT ACA-3'; Reverse: 5'-TGC AAC ACC TTT CTG ACC TTT GG-3') were designed to amplify cDNA of less than 200 bp. Final reaction volume was 20 μl including 2 μl of cDNA, 10 μl of 2 × premix, 2 μl of each primer, 0.4 μl of ROX and 3.6 μl of DEPC-treated ddH_2O. PCR conditions were 95℃ for 10 min followed by 40 cycles of 95℃ for 30 sec, 60℃ for 30 sec and 72℃ for 30 sec. Data were analyzed using Applied Biosystems software. The results were reported as the expression relative to the level detected on Day 12 of the estrous cycle after normalization of the transcript amount to the endogenous *RPL7* control by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

5. Statistical Analyses

Data from real-time RT-PCR analysis for *KL* mRNA levels were subjected to least squares ANOVA using the General Linear Models procedures of SAS (Cary, NC). As sources of variation, the model included day, pregnancy status (cyclic or pregnant, Days 12 and 15 post-estrus), and the interactions of these two factors to evaluate the steady-state levels of *KL* mRNAs. Effects of day of pregnancy (Day 12, Day 15, Day 30, Day 60, Day 90 and Day 114) in the endometrium and effects of day of pregnancy (Day 30, Day 60, Day 90 and Day 114) in chorioallantoic tissue for data from real-time RT-PCR for *KL* expression, and data from IL1B dose-response studies were analyzed by least squares regression analysis. To evaluate the effect of steroid hormones on *KL* mRNA levels, the model included treatment and animal as sources of variation. Preplanned contrasts (control vs. E_2; control vs. P_4; E_2 vs. E_2+P_4; E_2+P_4 vs. E_2+P_4+ICI and E_2+P_4 vs. E_2+P_4+RU486) were used to test for effects of treatments in the explant cultures. Data are presented as least squares means with standard error. Data from real-time RT-PCR analysis of *KL* mRNA levels between Non-NT and NT were subjected to the Student *t*-test procedure of SAS (Cary, NC), and are presented as means with SEM. A *P* value of 0.05 or less was considered significant, whereas a *P* value of 0.05 to 0.10 was considered a trend toward significance.

RESULTS

1. Expression of *KL* mRNA in the Uterine Endometrium during the Estrous Cycle and Pregnancy in Pigs

To determine whether *KL* mRNA is expressed in the uterine endometrium during the estrous cycle and pregnancy in pigs, real-time RT-PCR analysis was performed using the endometrial tissues from cyclic and pregnant gilts. As shown in Fig. 1, steady-state levels of endometrial *KL* mRNA were affected by day, but not by status and their interaction, on Day 12 to Day 15 post-estrus ($P<0.05$), and its expression during pregnancy showed a biphasic manner with high levels on Day 12 and Day 90 of pregnancy (linear effect of day, $P<0.05$).

2. Expression of *KL* mRNA Conceptuses on Days 12 and 15 of Pregnancy and Chorioallantoic Tissues during later Stage Pregnancy

Next, we asked if *KL* mRNA is expressed in porcine conceptuses during early pregnancy. We performed RT-PCR analysis using conceptuses by flushing the uterus from gilts on Days 12 and 15 of pregnancy. As shown in Fig. 2(A), *KL* mRNA was detected in conceptuses on Day 12 and Day 15 of pregnancy. To determine whether *KL* mRNA is expressed in chorioallantoic tissues during later stage pregnancy, real-time RT-PCR analysis was performed. *KL* mRNA was expressed in chorioallantoic tissues from Day 30 to term pregnancy, and its levels tended to increase toward term pregnancy (linear effect of day, $P=0.0603$) (Fig. 2(B)).

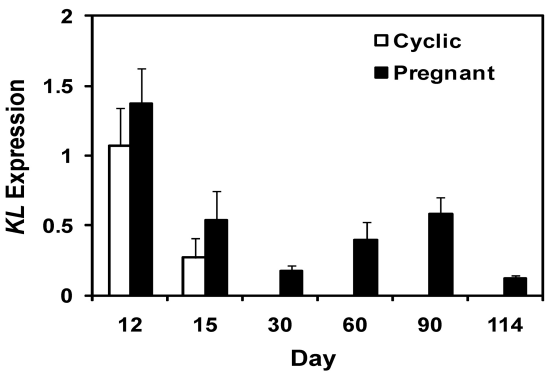
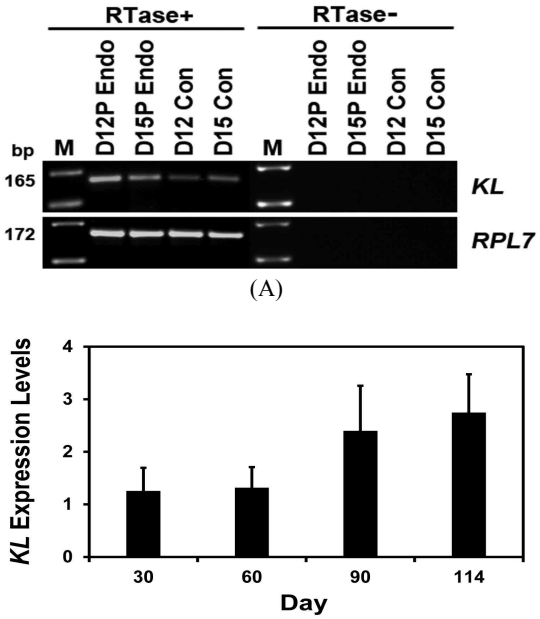


Fig. 1. Analysis of *KL* mRNA levels in the uterine endometrium by real-time RT-PCR analysis during the estrous cycle and pregnancy in pigs. Endometrial tissue samples from cyclic and pregnant gilts (n = 3～6 per day) were tested. Abundance of mRNA is presented as the expression relative to the level of *KL* mRNA measured on Day 12 of the estrous cycle after normalization of the transcript amount to *RPL7* mRNA. Data are presented as least squares means with standard error.

3. Effect of Steroid Hormones and IL1B on *KL* mRNA Levels in the Uterine Endometrium in Pigs

Because many uterine genes are regulated by estrogen and progesterone during the implantation period (Bazer *et al.*, 1998), the effect of E_2 and P_4 on *KL* mRNA levels in the uterine endometrium on Day 12 of pregnancy was investigated by real-time RT-PCR analysis. Uterine endometrial explant tissues from gilts on Day 12 of the estrous cycle were treated with control, E_2, P_4, E_2+P_4, E_2+P_4+ICI or E_2+P_4+RU486. As shown in Fig. 3(A), Levels of *KL* mRNA in the uterine endometrial explant tissues were not regulated by steroid hormones (P>0.05).

In addition to steroid hormone, IL1B is secreted from elongating conceptuses and affects expression of various genes of the uterine endometrium during the implantation period (Jaeger *et al.*, 2001; Ross *et al.*, 2003). To determine if levels of *KL* mRNA are regulated by IL1B in the uterine endometrium during the implantation period, the uterine endometrial explant tissues are treated with 0, 1, 10 or 100 ng/ml IL1B in the presence of both E_2 and P_4. Levels of *KL* mRNA tended to be up-regulated by treatment increasing doses of IL1B (linear effect of dose, P=0.0776) (Fig. 3(B)).

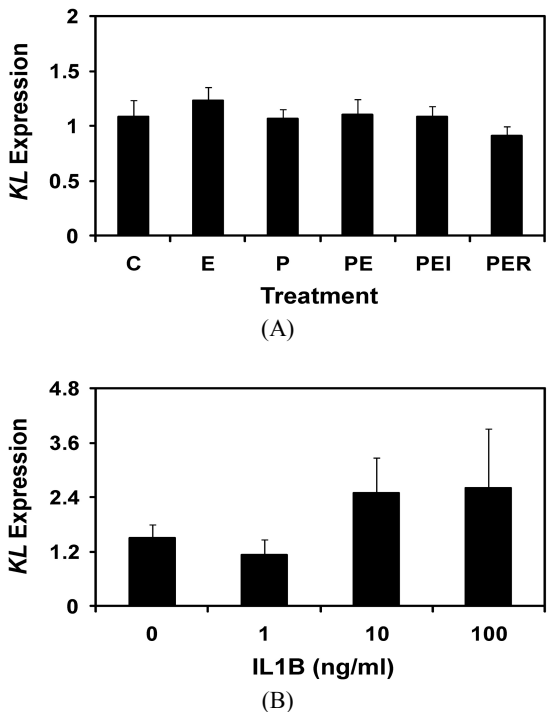


(A)

(B)

Fig. 2. Expression of *KL* mRNA by conceptuses from Days 12 and 15 of pregnancy (A) and by chorioallantoic tissues (B) during later stage pregnancy. (A) RT-PCR analysis of KL expression in conceptuses. *RPL7* cDNA was used as a loading control. RTase +/–, with (+) or without (–) reverse transcriptase; M, molecular marker; PBMC, peripheral blood mononuclear cells; D12P Endo, endometrium on Day 12 of pregnancy; D15P Endo, endometrium on Day 15 of pregnancy; D12 Con, Day 12 conceptus; D15 Con, Day 15 conceptus. (B) Real-time RT-PCR analysis of *KL* mRNA in chorioallantoic tissue samples on Day 30, Day 60, Day 90 and Day 114 of pregnancy. Data are reported as expression relative to that detected on Day 30 of pregnancy after normalization of the transcript amount to the endogenous *RPL7* control, and data are presented as least squares means with standard errors.

4. Comparison of *KL* mRNA Levels in the Uterine Endometrium with SCNT-derived Embryos to Those with Natural Mating-derived Embryos on Day 12 of Pregnancy in Pigs



(A)

(B)

Fig. 3. Effects of steroid hormones, estrogen and progesterone, (A) and IL1B (B) on expression of *KL* mRNA in porcine endometrial explant cultures by real-time PCR analysis. Endometrial explants from gilts on Day 12 of the estrous cycle were cultured in DMEM/F-12 in the presence of control (C), E_2 (E; 50 ng/ml), P_4 (P; 3 ng/ml), E_2+P_4, E_2+P_4+ ICI182,780 (I; 100 ng/ml, an estrogen receptor antagonist) or E_2+P_4+RU486 (R; 30 ng/ml, a progesterone receptor antagonist), and increasing doses of IL1B (0 ng/ml, 1 ng/ml, 10 ng/ml or 100 ng/ml) at 37℃ for 24 h. All experiments were repeated in triplicate with endometrium from each of the three gilts. Data are presented as least squares means with standard errors.

To determine whether expression of *KL* mRNA in the uterine endometrium on Day 12 of pregnancy in pigs is influenced by SCNT procedure, comparison of *KL* mRNA levels in the uterine endometrium from gilts carrying SCNT-derived conceptuses to those carrying conceptuses derived from natural mating on Day 12 of pregnancy was performed using real-time RT-PCR analysis. As shown in Fig. 4, levels of *KL* mRNA in the uterine endometrium from gilts with SCNT-derived conceptuses were significantly lower than those with conceptuses derived from natural mating ($P<0.05$).

DISCUSSION

This study investigated the expression and regulation of *KL* mRNA in the uterine endometrium during the estrous cycle and pregnancy in pigs and is the first investigation that identifies uterine *KL* expression in pigs. It was shown that 1) *KL* mRNA was expressed in the uterine endometrium during the estrous cycle and pregnancy in a pregnancy stage-specific manner; 2) *KL* mRNA was also expressed in conceptuses during early pregnancy and in chorioallantoic tissues during the later stage pregnancy; 3) expression of endometrial *KL* mRNA was not regulated by steroid hormones, estrogen and progesterone, but tended to be increased by IL1B during the implantation period; and 4) levels of *KL* mRNA on Day 12 of pregnancy were affected by the SCNT procedure in pigs.

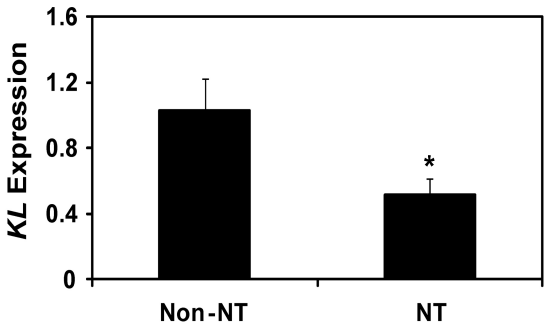


Fig. 4. Analysis of *KL* mRNA levels in the uterine endometrium from gilts with conceptuses generated by natural mating (non-NT) and those from gilts with SCNT-derived conceptuses (NT) on Day 12 of pregnancy using real-time RT-PCR analysis. Abundance of mRNA is presented as expression relative to that for *KL* mRNA in uterine endometria from gilts with non-NT conceptuses after normalization of transcript amounts to *RPL7* mRNA. Data are presented as least squares means with standard error. * $P<0.05$.

Conceptus in pigs undergoes the morphological change from spherical to tubular and then to filamentous forms, and elongated conceptuses secretes estrogen from trophectoderm cells into uterine lumen during the peri-implantation period in pigs (Bazer and Thatcher, 1977). Geisert and his colleagues (1982) have determined that conceptus-derived estrogen leads to increase of Ca^{2+} levels in the uterine lumen between Days 11 and 12 of pregnancy, and luminal Ca^{2+} levels are decreased immediately. It suggests that levels of Ca^{2+} in the uterus are tightly regulated during this period in pigs. Ca^{2+} is essential for the cell-to-cell adhesion between the trophectoderm and maternal endometrial epithelial cells during the implantation period (Wang *et al.*, 2002; Jha *et al.*, 2006; Li *et al.*, 2008). Our previous study showed that expression of TRPV6 is increased by estrogen of concept us origin during the implantation period in pigs (Choi *et al.*, 2009). In the present study, the highest levels of *KL* mRNA were coincident with increases in both luminal Ca^{2+} levels and endometrial TRPV6 expression on Day 12 of pregnancy in pigs. It provides the possibility that immediate decreases in luminal Ca^{2+} levels are induced by endometrial Ca^{2+} absorption via increased TRPV6 on the cell surface, and endometrial TRPV6 levels on Day 12 of pregnancy are maintained by Ca^{2+} channel accumulating action of KL during pregnancy in pigs.

Approximately 30 g of Ca^{2+} is transferred to placental layer for proper fetal development during successful pregnancy (Lafond *et al.*, 2001). Allantoic fluid Ca^{2+} concentration increases from Day 30 to Day 80 of pregnancy, and remains unaltered relatively to Day 100 of pregnancy in pigs. *TRPV6* are expressed in the uterine epithelium consistently and in the chorionic epithelium during later stage pregnancy in pigs (Choi *et al.*, 2009) and contributes Ca^{2+} transport from mother to developing fetus (Hoenderop *et al.*, 2002; 2005). *KL* was expressed in the uterine endometrium with increased levels on Day 90 of pregnancy and also in the chorioallantoic tissues during the later stage pregnancy in pigs, suggesting that *KL* of endometrial and placental origin may accumulate TRPV6 channels on the surface of the uterine epithelial and chorionic epithelial cells and aid to transport Ca^{2+} at the maternal-fetal interface in pigs.

KL protein has two forms, membrane-bound and secreted forms (Imura *et al.*, 2004), and only sKL carrying β-glucuronidase activity can regulates the activity of TRPV6 directly in physiological state (Lu *et al.*, 2008). Soluble sKL is generated by shedding of ectodomain in the extracellular domain including

two repeats, KL1 and KL2, of membrane-bound KL by membrane anchored-protease, ADAM10 and ADAM17 (Kuro-o *et al.*, 1997; Mian, 1998; Chen *et al.*, 2007). Although expression of ADAM10 and ADAM17 has not investigated in the porcine uterine endometrium, it has been identified that these membrane anchored-proteases are expressed in uterine endometrium and play a role in the uterine endometrial remodeling during pregnancy (Kim *et al.*, 2005; Kim *et al.*, 2006), suggesting that release of sKL by ADAM10 and ADAM17 may occur in the uterine endometrium during pregnancy in pigs. However, further study for the presence of sKL, ADAM10 and ADAM17 in porcine uterus is needed.

Membrane-bound KL also affects the control of calcium homeostasis. Membrane-bound KL functions as a canonical co-receptor for FGF23 with FGFR in kidney (Kuro-o, 2006; Urakawa *et al.*, 2006). When FGF23 complexes with FGFR and membrane-bound KL on the surface of renal epithelial cells, FGF23 signaling pathway mediated by membrane-bound KL in mouse kidney participates in a negative feedback mechanism to inhibit synthesis of $1,25(OH)_2D_3$, the active form of vitamin D_3, via suppressing expression of *Cyp27b1* gene that encodes 1α-hydroxylase inducing activation of vitamin D_3 (Shimada *et al.*, 2004). Disruption of this action causes increased serum levels of $1,25(OH)_2D_3$ (Yoshida *et al.*, 2002). As increased serum $1,25(OH)_2D_3$ levels up-regulates expression of calcium regulatory molecules including TRPV6, S100G, ATP2B1 and SLC8A1, the intestinal dietary Ca^{2+} absorption andrenal Ca^{2+} reabsorption are stimulated (den Dekker *et al.*, 2003; van de Graaf *et al.*, 2006), resulting in hypercalcemia (Yoshida *et al.*, 2002). This indirect effect of membrane-bound KL has not been determined in the uterus during pregnancy. In pigs, the FGFR signaling pathway stimulates proliferation and migration of conceptus trophectoderm and chorionic epithelial cells (Ka *et al.*, 2000; 2001; 2007), and local expression of CYP27B1 in the uterine endometrium and the placenta during pregnancy (unpublished data, Y. Choi and H. Ka), suggesting that the indirect calcium regulatory mechanism of membrane-bound KL may occur in the uterus and the placenta during pregnancy in pigs.

The SCNT technique generating cloned animals is a useful technique, but the efficiency to produce viable offspring is still very low (Campbell *et al.*, 2005). It has been suggested that abnormal extra-embryonic tissue formation (Kim *et al.*, 2005; Chae *et al.*, 2006; Jouneau *et al.*, 2006) and inappropriate uterine responsiveness to the developing conceptuses (Ka *et al.*, 2008; Kim *et al.*, 2009) result in the low efficiency of the SCNT technique. We compared *KL* expression levels in the uterine endometrium of gilts with SCNT-derived conceptuses to those of gilts with conceptuses generated by natural mating to determine whether regulation of calcium concentration by calcium regulatory molecules in the uterus was affected by the SCNT procedure. This study showed that expression of *KL* mRNA on Day 12 of pregnancy was decreased in the uterine endometrium from gilts with SCNT-derived conceptuses compared to that with conceptuses generated by natural mating. Our previous study has shown that secretion of estrogen and IL1B from conceptus is significantly reduced in the uterus carrying SCNT-derived conceptuses on Day 12 of pregnancy (unpublished result). Since that IL1B tended to increase expression of *KL* in the uterine endometrium, it is likely that inadequate levels of IL1B in the uterus with SCNT-derived conceptuses induce decreased endometrial expression of *KL* and, in turn, cause low efficiency in viable cloned pig production.

This study in pigs demonstrated that *KL* mRNA was expressed in the uterine endometrium during the estrous cycle and pregnancy, in conceptuses during early pregnancy, and in chorioallantoic tissues during later stage pregnancy. Taken together, these results suggest that KL may play an important role in the control of Ca^{2+} levels by regulating $1,25(OH)_2D_3$ synthesis and activity of calcium regulatory molecules for the establishment and maintenance of pregnancy in pigs. Further studies elucidating the regulatory mechanisms of KL in control of both $1,25(OH)_2D_3$ synthesis and TRPV6 activity in the uterus are needed to understand the role of KL in the uterine endometrium during pregnancy in pigs.

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