



## Activated Leukocyte Cell Adhesion Molecule: Expression in the Uterine Endometrium during the Estrous Cycle and Pregnancy in Pigs\*

Mingoo Kim, Jangsoo Shim, Heewon Seo, Yohan Choi, Chang-Kyu Lee<sup>1</sup>, and Hakhyun Ka\*\*

Division of Biological Science and Technology, IPAID and Institute of Biomaterials,  
Yonsei University, Wonju, 220-710, Korea

**ABSTRACT** : The pig exhibits true epitheliochorial placentation, where the fetal membrane maintains attachment throughout pregnancy but does not invade into the maternal uterine endometrium. Accordingly, the expression and function of cell adhesion molecules are very important for embryo implantation and the establishment of pregnancy. In our recent microarray analysis, we found that activated leukocyte cell adhesion molecule (ALCAM) was expressed in the uterine endometrium during pregnancy in pigs. To better understand the roles of ALCAM in the establishment and maintenance of pregnancy, we examined *ALCAM* expression in the uterine endometrium during the estrous cycle and pregnancy in pigs. Real-time RT-PCR analysis showed that *ALCAM* was differentially expressed in the uterine endometrium during the estrous cycle and pregnancy, with the highest levels on D12 of pregnancy. *ALCAM* mRNA was localized to the luminal and glandular epithelial cells and to the trophoctoderm of conceptuses during early pregnancy. The steroid hormones estrogen and progesterone had no effect on *ALCAM* expression in an endometrial explant culture study. Further, we found that *ALCAM* expression in the uterine endometrium from gilts with somatic cell nuclear transfer-derived embryos was not different from that in gilts with embryos from natural mating. *ALCAM* was expressed in a pregnancy stage- and cell type-specific manner in the uterine endometrium and conceptuses during pregnancy. These findings suggest that ALCAM may play a role in the establishment of pregnancy. Further analysis of ALCAM will provide insight into the implantation process and establishment of pregnancy in pigs. (**Key Words** : Pig, Pregnancy, Uterus, Endometrium, ALCAM)

### INTRODUCTION

The establishment and maintenance of pregnancy requires complex and well-organized interactions between the implanting embryos and the maternal uterine endometrium. In pigs, embryo implantation begins around day (D) 12 of pregnancy. During the implantation period, trophoblastic elongation, maternal recognition of pregnancy, and embryo attachment occur at the fetal-maternal interface

(Bazer et al., 2009). Pigs exhibit true epitheliochorial placentation, where the fetal membrane does not invade into the uterine endometrium but superficially interdigitates with the luminal epithelial layer. Thus, cell-cell adhesion between the conceptus trophoctoderm and endometrial luminal epithelial cells is the final step for placentation, and various cell adhesion molecules are involved in this process (Burghardt et al., 1997; Burghardt et al., 2002). For example, SPP1 (secreted phosphoprotein 1, also called osteopontin) promotes trophoctodermal cell migration and adhesion with luminal epithelial cells by interaction with integrin (Erikson et al., 2009). Integrin subunits, receptors for extracellular matrix (ECM) proteins such as SPP1, fibronectin, and vitronectin, are expressed in the conceptuses and luminal epithelial cells for cell-to-cell attachment during the implantation period (Bowen et al., 1996; Burghardt et al., 1997). Down-regulated mucin 1 (MUC1) expression exposes integrins localized in the apical surface of the uterine luminal epithelial cells and facilitates binding of integrins to ECM proteins at the fetal-maternal

\* This study was supported by the BioGreen 21 Program (#20070301034040; #20070401034031), Rural Development Administration, and the National Research Foundation (NRF-2010-413-B00024) funded by the Korean Government, Republic of Korea.

\*\* Corresponding Author : H. Ka. Tel: +82-33-760-2369, Fax: +82-33-760-2186, E-mail: hka@yonsei.ac.kr

<sup>1</sup> Department of Agricultural Biotechnology, Research Institute for Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Korea.

Received February 9, 2011; Accepted April 3, 2011

interface (Burghardt et al., 2002). Failures of these serial events and subsequent abnormal maternal-fetal interactions lead to early embryonic loss in pigs (Pope, 1994; Bazer et al., 2009; Kim et al., 2009), but the process of initial cell-to-cell attachment and maintenance of cell adhesion during pregnancy is not fully understood.

Activated leukocyte cell adhesion molecule (ALCAM; also known as CD166) is a glycoprotein that acts as a cell surface ligand for CD6 (Ohneda et al., 2001). ALCAM interacts not only with CD6 in a heterotypic manner, but also homotypically with itself (Bowen et al., 1996; Cayrol et al., 2008). ALCAM is expressed in a variety of cells, including fibroblasts (Singer et al., 1997), pancreatic acinar and islet cells (Stephan et al., 1999), mesenchymal stem cells (Bruder et al., 1998; Arai et al., 2002), neurons (Konno et al., 2001), and trophectodermal cells (Fujiwara et al., 2003). Expression of ALCAM in trophectodermal cells is particularly critical for initial attachment of human embryos (Fujiwara et al., 2003), because ALCAM-ALCAM interactions form a tight ALCAM network between cells (Bowen et al., 1995). ALCAM expression is induced by cytokines such as interleukin-3 (IL3), macrophage-colony stimulating factor (M-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF)- $\alpha$ , and interferon- $\gamma$  (IFNG) (Levesque et al., 1998; Cayrol et al., 2008). In pigs, the uterine endometrial gene expression is influenced by various factors such as progesterone of ovarian origin and estrogen, interleukin-1 $\beta$  (IL1B), and IFNG of conceptus origin during early pregnancy (Lefevre et al., 1990; Burghardt et al., 1997; Geisert and Yelich, 1997; Ross et al., 2003; Bazer et al., 2009). Using microarray analysis, we have found that ALCAM is expressed in the uterine endometrium during pregnancy (Kim and Ka, manuscript in preparation), but the expression and functions of ALCAM in the uterine endometrium during the estrous cycle and pregnancy in pigs are not well understood. In addition, regulatory mechanism of ALCAM expression in the uterine endometrium needs to be elucidated.

The efficiency to produce viable offspring using somatic cell nuclear transfer (SCNT) technique is extremely low in pigs (Keefer, 2008), and is influenced by several factors, including quality of recipient oocytes, epigenetic reprogramming status of donor nucleus, and SCNT procedures (Humpherys et al., 2001; Campbell et al., 2005; Farin et al., 2006). Appropriate interaction between the conceptus and the uterine endometrium is required for normal uterine function to support fetal development (Bazer et al., 2009; Ashworth et al., 2010), but the interaction between the SCNT embryo and the maternal uterus is impaired in pigs carrying SCNT embryos (Chae et al., 2008; Bauersachs et al., 2009; Kim et al., 2009). Our recent studies have shown that many uterine endometrial genes

related to cell-to-cell interaction are dysregulated in the uterine endometrium with SCNT embryos on D30 (Ka et al., 2008) and term pregnancy (Kim et al., 2009). Accordingly, it would be interesting to know whether endometrial ALCAM is appropriately expressed in the uterus with SCNT embryo on D12 of pregnancy.

Therefore, to better understand the role of ALCAM at the fetal-maternal interface in pigs during pregnancy, we determined i) the pattern of ALCAM expression in the uterine endometrium during the estrous cycle and pregnancy; ii) localization of ALCAM mRNA in the uterine endometrium; iii) effects of steroid hormones on ALCAM mRNA expression; and iv) ALCAM expression levels in the uterine endometrium from gilts with embryos derived from SCNT compared with those from gilts with embryos from natural mating.

## MATERIALS AND METHODS

### Recovery and *in vitro* maturation of oocytes

Recovery and *in vitro* maturation of oocytes, SCNT, and embryo transfer were conducted as described in Ka et al. (2008). In brief, cumulus-oocyte complexes (COCs) were collected by aspiration from ovarian antral follicles of 3.0-6.0 mm in diameter from ovaries collected at a local slaughterhouse. COCs having at least three layers of compacted cumulus cells were selected and cultured in tissue culture medium (TCM)-199 (Invitrogen, Carlsbad, CA) supplemented with 0.6 mM cysteine, 10 ng/ml epidermal growth factor, 4 IU/ml equine chorionic gonadotropin (eCG; Intervet, Boxmeer, The Netherlands) and human chorionic gonadotropin (hCG; Intervet), and 10% (v/v) porcine follicular fluid. The COCs were cultured for 22 h with hormones and then for 22 h without hormones in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 38.5°C.

### SCNT

After *in vitro* maturation, cumulus cells were removed by repeatedly pipetting for 1 min in a tyrode-lactate-HEPES (TL-HEPES) medium supplemented with 0.1% (w/v) hyaluronidase (Hagen et al., 1991). A cumulus-free oocyte was held with a holding micropipette and the zona pellucida was partially incised with a fine glass needle to make a slit near the first polar body. The first polar body and adjacent cytoplasm of the oocyte were extruded by squeezing with the same needle (Hyun et al., 2003). Oocytes were enucleated in TL-HEPES supplemented with 0.3% BSA and 7.5 mg/ml cytochalasin B. The cultured porcine embryonic fibroblast cells as nuclear donors were used after cell cycle synchronization by serum starvation for 48 h. A donor cell was inserted into the perivitelline space of an enucleated oocyte. Donor cell-oocyte complexes were

placed in a 280 mM mannitol solution containing 0.5 mM HEPES, 0.1 mM CaCl<sub>2</sub> and 0.1 mM MgCl<sub>2</sub> for 2 min and transferred to a chamber consisting of two electrodes overlaid. They were fused and activated simultaneously with a DC pulse (2.0 kV/cm 30 μs×1) using the BTX Electro-cell Manipulator 2001 (BTX, San Diego, CA). The reconstructed oocytes were then cultured in modified NCSU23 (Petters and Wells, 1993) supplemented with 4 mg/ml BSA in a humidified atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> at 38.5°C.

#### Animals, embryo transfer, and tissue collection

All experimental procedures involving animals were conducted in accordance with the Guide for the 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 pigs were assigned randomly to either cyclic or pregnant status. Twenty-four gilts were hysterectomized on D12 and D15 of the estrous cycle and D12, D15, D30, D60, D90, or D114 of pregnancy (n = 3 gilts/day/status). Pregnancy was confirmed by the presence of apparently normal conceptuses with filamentous morphology in uterine flushings or fetuses. Endometrium dissected from the myometrium was collected from two different areas of the middle portion of each uterine horn.

Endometrial tissue samples from gilts with embryos generated by SCNT on D12 of pregnancy were obtained as described in Ka et al. (2008). Crossbred prepubertal gilt weighing between 100 and 105 kg was used as the recipient of the embryos. To induce estrus in the gilt, 1,000 IU of eCG were injected intramuscularly, followed by an injection of 1,500 IU hCG 72 h later. The embryos produced by SCNT were cultured for 1 or 2 days. A total of 150 embryos that were morphologically normal at the 1-cell or 2-4-cell stages were selected for transfer. Embryos were then transferred into the oviducts of the recipient gilt approximately 48 h after hCG injection. Uterine endometrial tissues on D12 of pregnancy were obtained from four gilts with SCNT embryos and three gilts with embryos by natural mating (Non-NT). Endometrial tissues were snap-frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction. For *in situ* hybridization and immunohistochemistry, cross-sections of endometrium and conceptuses were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 24 h and then embedded in paraffin, as previously described (Ka et al., 2000).

#### Explant culture

Endometrium dissected from the myometrium was placed into warm phenol red-free Dulbecco's Modified Eagle Medium/F-12 culture medium (DMEM/F-12; Sigma,

St. Louis, MO) containing penicillin G (100 IU/ml) and streptomycin (0.1 mg/ml) as described previously (Ka et al., 2001), with some modifications. The endometrium was minced with scalpel blades into small pieces (2-3 mm<sup>3</sup>), and aliquots of 500 mg were placed into T25 flasks with serum-free modified DMEM/F-12 containing 10 μg/ml insulin (catalog number I5500; Sigma), 10 ng/ml transferrin (catalog number T1428; Sigma), and 10 ng/ml hydrocortisone (catalog number H0396; Sigma). Endometrial explants were cultured immediately after mincing in the presence of ethanol (control), estradiol-17β (E<sub>2</sub>; 50 ng/ml; catalog number E8875; Sigma), progesterone (P<sub>4</sub>; 3 ng/ml; catalog number P0130; Sigma), E<sub>2</sub>+P<sub>4</sub>, E<sub>2</sub>+P<sub>4</sub>+ICI182,780 (ICI; an estrogen receptor antagonist; 200 ng/ml; Tocris, Ballwin, MO), or E<sub>2</sub>+P<sub>4</sub>+RU486 (RU; a progesterone receptor antagonist; 30 ng/ml; catalog number M8046; Sigma) for 24 h with rocking in an atmosphere of 5% carbon dioxide in air at 37°C. Explant tissues were then harvested and total RNA was extracted for real-time RT-PCR analysis of *ALCAM* mRNA levels. These experiments were conducted using endometria from three individual gilts on D12 of the estrous cycle. Treatments were performed in triplicate on tissues obtained from each gilt.

#### RNA isolation

Total RNA from endometrial tissues was extracted using TRIzol (Invitrogen, Carlsbad, CA) and the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity and integrity of the total RNA were checked using a NanoDrop (NanoDrop Technologies, Wilmington, DE) and Experion (Bio-rad, Hercules, CA), respectively.

#### Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) for *ALCAM* analysis

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

Two micrograms of total RNA were treated with DNase I (Promega, Madison, WI) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) to obtain cDNAs. The cDNA templates were then diluted 1:5 with sterile water and amplified by PCR using Taq polymerase (Takara Bio, Shiga, Japan), and specific primers based on mRNA sequences of porcine *ALCAM* (GenBank accession number AJ311681; forward, 5'-CCT TCA GGT CCT CCA CAA AG -3'; reverse, 5'-ATG TGA TGT TGC CAT CTG GA-3') and porcine ribosomal protein L7 (*RPL7*),

(GenBank accession number NM\_001113217; 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 302 bp and 172 bp, respectively. PCR conditions for *ALCAM* were 35 cycles of 94°C for 15 s, 60°C for 20 s, and 72°C for 1 min. PCR products 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 into a pCRII vector (Invitrogen).

### Quantitative real-time RT-PCR

To analyze levels of *ALCAM* mRNA in the uterine endometrium, real-time RT-PCR was performed using the Applied Biosystems StepOnePlus System (Applied Biosystems, Foster City, CA) and the SYBR Green method. Complementary DNAs were synthesized from 4 µg of total RNA isolated from different uterine endometrial tissues, and newly synthesized cDNAs (total volume of 21 µl) were diluted 1:4 with sterile water and then used for PCR. Specific primers based on porcine *ALCAM* (GenBank accession number AJ311681; forward, 5'-CCA GAA TAC AAA AAC AGA ATT GAG CC-3'; reverse, 5'-TAG ATG GTT GCT TGG GAC ACC TTG AC-3') and porcine *RPL7* (GenBank accession number NM\_001113217; 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 150 bp and 172 bp, respectively. Power SYBR Green PCR Master Mix (Applied Biosystems) was used for the PCR reaction. 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 dH<sub>2</sub>O. PCR conditions were 95°C for 15 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The results were reported as the expression relative to the level detected on D12 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).

### Non-radioactive *in situ* hybridization

Non-radioactive *in situ* hybridization was performed as described previously with some modifications (Braissant and Wahli, 1998). Sections (5 µm thick) were rehydrated through successive baths of xylene, 100% ethanol, 95% ethanol, diethylpyrocarbonate (DEPC)-treated water, and DEPC-treated PBS. Tissue sections were permeabilized with DEPC-treated PBS containing 0.3% Triton X-100 for 15 min. After washing in DEPC-treated PBS, they were digested with 5 µg/ml Proteinase K (Sigma) in TE (100 mM Tris-HCl, 50 mM EDTA, pH 7.5) at 37°C. After postfixation in 4% paraformaldehyde, sections were

incubated twice for 15 min each in PBS containing 0.1% active DEPC, and equilibrated for 15 min in 5X SSC. The sections were prehybridized for 2 h at 68°C in hybridization mix (50% formamide, 5X SSC, 500 µg/ml herring sperm DNA; 200 µl on each section). Sense and antisense *ALCAM* riboprobes labeled with DIG-UTP were denatured for 5 min at 80°C and added to the hybridization mix. The hybridization reaction was carried out at 68°C overnight. Prehybridization and hybridization were performed in a box saturated with a 5X SSC - 50% formamide solution to avoid evaporation, and no coverslips were used. After hybridization, sections were washed for 30 min in 2X SSC at room temperature, 1 h in 2X SSC at 65°C, and 1 h in 0.1X SSC at 65°C. Probes bound to the section were immunologically detected 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 a chromogenic substrate, according to the manufacturer's protocol (Roche, Indianapolis, IN).

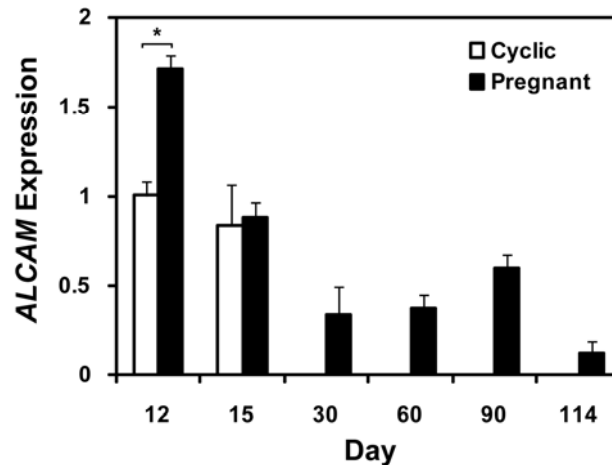
### Statistical analysis

Data from real-time RT-PCR for *ALCAM* expression 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), and their interactions to evaluate the steady-state level of *ALCAM* mRNA, and treatment and animal to evaluate the effect of steroid hormones on *ALCAM* mRNA. Preplanned contrasts (control vs. E<sub>2</sub>; control vs. P<sub>4</sub>; E<sub>2</sub> vs. E<sub>2</sub>+P<sub>4</sub>; E<sub>2</sub>+P<sub>4</sub> vs. E<sub>2</sub>+P<sub>4</sub>+ICI) for *ALCAM* expression were applied to test for effects of treatments in the explant cultures. Data are presented as least squares means with SE (standard error). Data from real-time RT-PCR analysis for comparison of *ALCAM* levels in the endometrium with SCNT and Non-NT embryos were subjected to *t* tests, and are presented as means with SE.

## RESULTS

### Expression and regulation of *ALCAM* mRNA in the uterine endometrium during the estrous cycle and pregnancy in pigs

To determine *ALCAM* mRNA levels in the uterine endometrium during the estrous cycle and pregnancy in pigs, we conducted real-time RT-PCR analysis. As shown in Figure 1, relative levels of *ALCAM* mRNA were high during early pregnancy in the uterine endometrium and decreased after D15 of pregnancy (day,  $p < 0.05$ ). *ALCAM* mRNA levels in the uterine endometrium on D12 of pregnancy were higher than those on D12 of the estrous cycle (day x status,  $p < 0.05$ ).

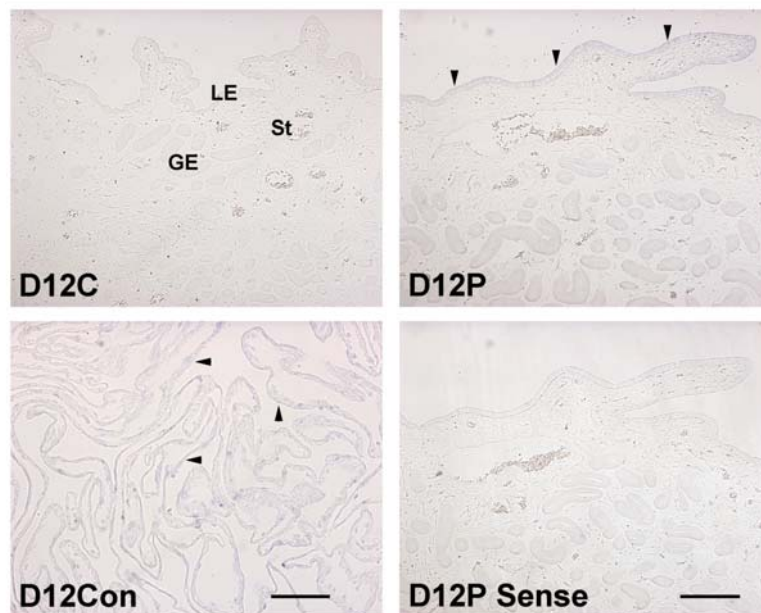


**Figure 1.** Analysis of *ALCAM* mRNA levels in the uterine endometrium during the estrous cycle and pregnancy in pigs by real-time RT-PCR analysis. Endometrial tissue samples from cyclic and pregnant gilts ( $n = 3$  per day) were tested. Abundance of mRNA is presented as the expression relative to the level of *ALCAM* measured on D12 of the estrous cycle after normalization of the transcript amount to *RPL7*. *ALCAM* mRNA levels were highest on D12 during pregnancy (day,  $p < 0.05$ ), and the levels of *ALCAM* mRNA on D12 of pregnancy were significantly higher than those on D12 of the estrous cycle (day  $\times$  status,  $p < 0.05$ , indicated as asterisk). Data are presented as least squares means with standard error.

#### Localization of *ALCAM* mRNA in the uterine endometrium during the estrous cycle and pregnancy

To determine which cell types express *ALCAM* mRNA in the uterine endometrium during the estrous cycle and pregnancy and in conceptuses, we performed *in situ* hybridization analysis using uterine endometrial tissues from D12 and D15 of the estrous cycle and pregnancy and

conceptuses from D12 of pregnancy. *ALCAM* mRNA was mainly localized in the luminal and glandular epithelial cells in the uterine endometrium during the estrous cycle and pregnancy (Figure 2). The signal intensity of *ALCAM* mRNA was stronger in the uterine endometrium on D12 of pregnancy than that on D12 of the estrous cycle, which was consistent with real-time RT-PCR results. In addition, the



**Figure 2.** *In situ* hybridization analysis of *ALCAM* mRNA in the uterine endometrium during the estrous cycle and pregnancy in pigs. A section from D12 of pregnancy hybridized with a DIG-labeled sense *ALCAM* cRNA probe (Sense) is shown as a positive and a negative control, respectively. *ALCAM* mRNA was primarily localized to the luminal and glandular epithelial cells of the uterine endometrium and trophoctoderm of conceptuses on D12 of pregnancy. D, day; C, estrous cycle; P, pregnancy; LE, luminal epithelium; St, stroma; Tr, trophoctoderm, Con; conceptus. Arrow heads indicate *ALCAM* transcript-positive signals on luminal epithelial cells and conceptus trophoctoderm cells. Scale bar = 100  $\mu$ m and 200  $\mu$ m in D12P Con.

signal intensity of *ALCAM* mRNA seemed to be slightly stronger in luminal compared to glandular epithelial cells. *ALCAM* mRNA was also detected in the trophoblast of conceptuses on D12 of pregnancy.

#### Expression of *ALCAM* in the conceptus during early pregnancy

To determine whether conceptuses express *ALCAM* mRNA during the implantation period, we analyzed *ALCAM* expression in conceptuses from D12 and D15 of pregnancy by RT-PCR and found *ALCAM* mRNA expression on both D12 and D15 of pregnancy (Figure 3).

#### Effect of steroid hormones on *ALCAM* expression

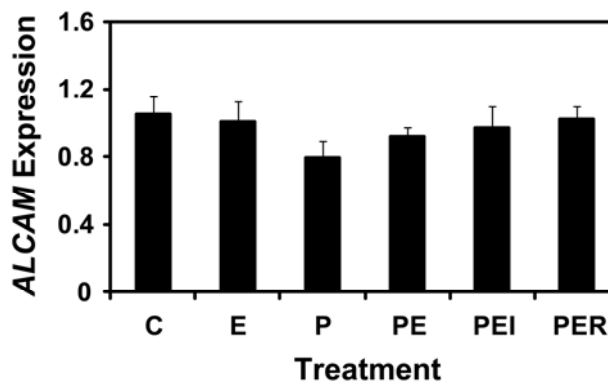
Because *ALCAM* expression was highest on D12 of pregnancy in the uterine endometrium, we hypothesized that *ALCAM* expression might be regulated by estrogen secreted by elongating conceptuses and progesterone of ovarian origin. To determine the effect of steroid hormones on *ALCAM* expression in the uterine endometrium, we treated explant cultures of uterine endometrial tissues from gilts on D12 of the estrous cycle with steroid hormones and inhibitors of those hormones receptors. Estrogen and progesterone did not affect *ALCAM* mRNA expression in the uterine endometrium (Figure 4).

#### Comparison of *ALCAM* expression levels in the uterine endometrium in pigs carrying Non-NT and NT-derived embryos

To determine influence of *ALCAM* expression on the establishment of pregnancy in recipients with SCNT embryos, we compared *ALCAM* mRNA expression levels in the uterine endometrium of pigs carrying Non-NT and SCNT-derived embryos by real-time RT-PCR. *ALCAM* levels were not significantly different depending on embryo type.

## DISCUSSION

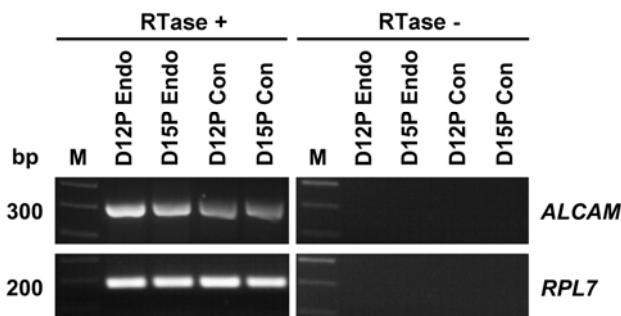
In the present study, we determined in pigs that i)



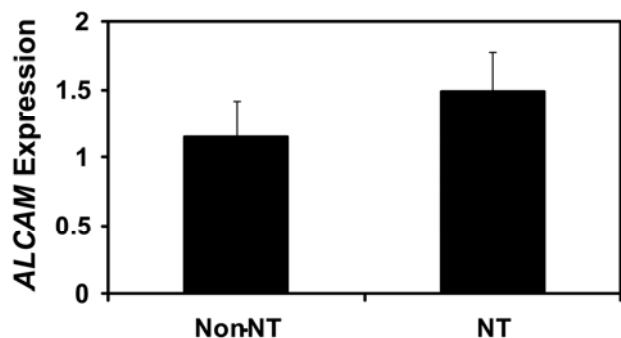
**Figure 4.** Effects of steroid hormones on *ALCAM* mRNA levels in endometrial explant cultures by real-time RT-PCR analyses. Endometrial explants from gilts on D12 of the estrous cycle were cultured in DMEM/F-12 in the presence of control (C),  $E_2$  (E; 50 ng/ml),  $P_4$  (P; 3 ng/ml),  $E_2+P_4$ ,  $E_2+P_4+ICI$  (I; 200 ng/ml, an estrogen receptor antagonist), or  $E_2+P_4+RU$  (R; 30 ng/ml, a progesterone receptor antagonist), at 37°C for 24 h. For each treatment, all experiments were repeated in triplicate with endometrium from each of three gilts. Data are presented as least squares means relative units with standard error.

*ALCAM* expression in the uterine endometrium changed during the estrous cycle and pregnancy with the highest levels on D12 of pregnancy; ii) *ALCAM* mRNA was localized in the luminal and glandular epithelial cells in the endometrium; iii) *ALCAM* expression was detected in conceptuses on D12 and D15 of pregnancy, localized to the conceptus trophoblast; iv) steroid hormones (estrogen and progesterone) did not regulate *ALCAM* expression in the uterine endometrium; and v) *ALCAM* expression levels in the uterine endometrium were not different between pigs carrying SCNT and Non-NT-derived embryos.

We observed that *ALCAM* expression was differentially regulated in the uterine endometrium during pregnancy in pigs. *ALCAM* expression was higher in the uterine endometrium during early pregnancy than in the mid- to late stages of pregnancy. In particular, *ALCAM* expression levels were highest on D12 of pregnancy, which is the period of maternal recognition of pregnancy in pigs (Bazer



**Figure 3.** Detection of *ALCAM* mRNA in the conceptus on D12 and 15 of pregnancy by RT-PCR analysis. *RPL7* was used as a positive control. RTase +/-, with (+) or without (-) reverse transcriptase; M, 100 bp molecular marker; D12 Endo, endometrium from Day 12 of pregnancy; D15 Endo, endometrium from Day 15 of pregnancy; D12 Con, Day 12 conceptus; D15 Con, Day 15 conceptus.



**Figure 5.** Analysis of *ALCAM* mRNA levels in the uterine endometrium of pigs with embryos derived from SCNT (NT) compared to embryos from natural mating (Non-NT) on D12 by real-time RT-PCR. Abundance of mRNA is presented as the expression relative to the levels of *ALCAM* mRNA measured in the uterine endometrium from gilts with non-NT embryos after normalization of the transcript amount to *RPL7* mRNA. Data are presented as means with standard error.

et al., 2009). Conceptuses during this period elongate to become filamentous forms and begin contact with the uterine endometrium for the establishment of pregnancy (Dantzer, 1985; Bazer et al., 2009). Cell-to-cell attachment between the trophoblast of conceptuses and uterine epithelial cells is critical for the establishment of pregnancy (Dantzer, 1985), and a variety of cell adhesion molecules are dynamically regulated during the implantation period in the uterine endometrium (Burghardt et al., 1997; Geisert and Yelich, 1997; Fernando et al., 2007; Song et al., 2010). Among them, integrins, selectins, galectins, heparan sulfate proteoglycans, heparin-binding EGF-like growth factors, cadherins, CD44, and ECM molecules are known to be associated with initial attachment of conceptuses to the endometrial epithelial cells (Burghardt et al., 1997; Bazer et al., 2009). Because *ALCAM*, a cell adhesion molecule, is also involved in cell-to-cell attachment by interacting with CD6 heterotypically or with *ALCAM* homotypically (Bowen et al., 1995; Ohneda et al., 2001) and its expression is high at the time of conceptus implantation, our result suggests that *ALCAM* may also be critical for cell-to-cell adhesion between the conceptus trophoblast and the uterine endometrium during the implantation process in pigs.

*ALCAM* was localized to the luminal and glandular epithelial cells and the trophoblast of conceptuses during early pregnancy. *ALCAM* has been detected in brain, heart, guts, bone marrow, thymus, and hematopoietic sites in humans (Patel et al., 1995; Uchida et al., 1997; Bruder et al., 1998; Cortes et al., 1999; Stephan et al., 1999; Konno et al., 2001; Gessert et al., 2008), as well as luminal epithelial cells during the secretory phase in the human placenta and in blastocysts (Fujiwara et al., 2003). In our study, localization of *ALCAM* expression in the luminal epithelial

cells and conceptus trophoblast suggest the possibility of homotypic interactions of *ALCAM* between the uterus and conceptuses. Localization of *ALCAM* mRNA in the glandular epithelial cells indicates that *ALCAM* may be secreted from glandular epithelial cells into the uterine lumen, since the soluble isoform of *ALCAM* is produced by lung microvascular endothelial cells in humans (Ikeda and Quertermous, 2004). Further study to determine if soluble *ALCAM* is present in the uterine lumen is needed.

*ALCAM* expression is regulated by different hormones and cytokines in various cell types. In MCF7 cells, estrogen increases *ALCAM* expression (Jeziarska et al., 2006), and human chorionic gonadotrophin induces *ALCAM* expression in the cumulus-oocyte complex of the human ovary (Hernandez-Gonzalez et al., 2006). GM-CSF induces *ALCAM* expression in CD14+ monocytes (Levesque et al., 1998). Co-treatment with TNF and IFNG induce *ALCAM* expression in human endothelial cells of the blood-brain barrier (Cayrol et al., 2008). We hypothesized that estrogen of conceptus origin induces *ALCAM* expression in the uterine endometrium for a number of reasons. First, *ALCAM* expression is induced by estrogen in MCF7 cells (Jeziarska et al., 2006). In addition, estrogen, a signal for the maternal recognition of pregnancy in pigs, is secreted from elongating conceptuses around D12 of pregnancy (Bazer et al., 2009). Finally, we observed that *ALCAM* expression was highest in the uterine endometrium on D12 of pregnancy. Because progesterone of ovarian origin is the major hormone affecting the uterine endometrium during pregnancy, we further postulated that progesterone affects *ALCAM* expression in the endometrium. We found, however, that estrogen and progesterone did not change *ALCAM* expression in the present study, indicating that factors other than estrogen and progesterone may be involved in *ALCAM* expression in the uterine endometrium in pigs. In the porcine uterus, elongating conceptuses secrete IL1B on D12 of pregnancy (Ross et al., 2003) and IFNG between D12 and D18 of pregnancy (Lefevre et al., 1990), and these cytokines may induce endometrial *ALCAM* expression. Another possibility is that the uterine endometrial tissues used in this study were from D12 of the estrous cycle and so had previous exposure to progesterone that minimized their response to *in vitro* treatment with estrogen and/or progesterone. Further study involving the influence of steroid hormones and cytokines on the regulation of *ALCAM* expression in the uterine endometrium of pigs during early pregnancy is still needed.

Early embryonic mortality reaches approximately 40% in pigs (Pope, 1994), and the efficiency of generating viable offspring using SCNT cloning is very low in pigs (Keefer, 2008). Unsynchronized interactions between the conceptuses and the maternal endometrium may lead to aberrant gene expression in the uterine endometrium, which

consequently leads to early embryonic loss during early pregnancy (Bauersachs et al., 2009; Bazer et al., 2009; Kim et al., 2009). Premature estrogen administration results in decreased expression of *ITIH2* in the uterine endometrium in pigs, which could be related to loss of uterine glycocalyx leading to degradation of conceptuses (Ashworth et al., 2010). Furthermore, it has been suggested that abnormal expression of cell adhesion molecules, including integrins and ECM proteins, and some growth factors in the uterine endometrium with SCNT embryos are related to early embryonic loss of cloned pig embryos (Chae et al., 2008; Kim et al., 2009). Our previous study showed that SPP1, retinol-binding protein, and fibroblast growth factor 7 were aberrantly expressed in the endometrium with SCNT embryos compared to embryos from natural mating at term (Kim et al., 2009). In this study, we also looked for differences in *ALCAM* expression in the endometrium with SCNT and Non-NT embryos and found no differences. These findings indicate that the SCNT procedure does not affect endometrial *ALCAM* expression in pigs carrying SCNT embryos and that *ALCAM* expression is not affected by the presence of the conceptuses for implantation initiation in pigs. Further study is needed to determine whether *ALCAM* expression is affected by the SCNT procedure during mid- to late-stage pregnancy and whether there is any crucial role for *ALCAM* in the maintenance of pregnancy for SCNT embryos after the implantation stage.

In the porcine uterus, the exact function of *ALCAM* has not yet been determined. Since *ALCAM* is involved in cell-to-cell attachment to regulate migration of lymphocytes and cancer cells (Masedunskas et al., 2006; Cayrol et al., 2008), development of bone marrow (Arai et al., 2002), morphogenesis and maintenance of cardiac cells (Gessert et al., 2008), and initiation of the human embryo implantation (Fujiwara et al., 2003), we speculate that *ALCAM* may also play an important role in cell-to-cell attachment for embryo implantation at the maternal-fetal interface. Indeed, *ALCAM* expression was detected in both endometrial epithelial cells and conceptus trophectoderm in this study, and it is well known that *ALCAM* interacts with itself homotypically to trigger oligomerization of *ALCAM*s on the cell surface between the cells, enhancing a tight *ALCAM* network (Bowen et al., 1995). Expression of CD6, a heterophilic receptor for *ALCAM* expressed mainly in lymphocytes, has not been determined in the uterine endometrium or conceptuses in pigs so far, but it is possible that *ALCAM* may recruit and activate endometrial lymphocytes.

In conclusion, our results provide evidence that *ALCAM* is expressed in the luminal and glandular epithelial cells and trophectoderm of conceptuses during the implantation period in pigs. These findings suggest that *ALCAM* may be involved in the initial contact between the conceptuses and uterine endometrial epithelial cells for the establishment of

implantation in pigs. These results provide valuable information on the mechanisms of the implantation process in the pig, which exhibits epitheliochorial placentation.

## REFERENCES

- Arai, F., O. Ohneda, T. Miyamoto, X. Q. Zhang and T. Suda. 2002. Mesenchymal stem cells in perichondrium express activated leukocyte cell adhesion molecule and participate in bone marrow formation. *J. Exp. Med.* 195(12):1549-1563.
- Ashworth, M. D., J. W. Ross, D. Stein, F. White and R. D. Geisert. 2010. Endometrial gene expression of acute phase extracellular matrix components following estrogen disruption of pregnancy in pigs. *Anim. Reprod. Sci.* 122:215-221.
- Bauersachs, S., S. E. Ulbrich, V. Zakhartchenko, M. Minten, M. Reichenbach, H. D. Reichenbach, H. Blum, T. E. Spencer and E. Wolf. 2009. The endometrium responds differently to cloned versus fertilized embryos. *Proc. Natl. Acad. Sci. USA* 106(14):5681-5686.
- Bazer, F. W., T. E. Spencer, G. A. Johnson, R. C. Burghardt and G. Wu. 2009. Comparative aspects of implantation. *Reproduction* 138(2):195-209.
- Bowen, J. A., F. W. Bazer and R. C. Burghardt. 1996. Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm *in vivo*. *Biol. Reprod.* 55(5):1098-1106.
- Bowen, M. A., D. D. Patel, X. Li, B. Modrell, A. R. Malacko, W. C. Wang, H. Marquardt, M. Neubauer, J. M. Pesando, U. Francke, and et al., 1995. Cloning, mapping, and characterization of activated leukocyte-cell adhesion molecule (*ALCAM*), a CD6 ligand. *J. Exp. Med.* 181(6):2213-2220.
- Braissant, O. and W. Wahli. 1998. A simplified *in situ* hybridization protocol using non-radioactively labeled probes to detect abundant and rare mRNAs on tissue sections. *Biochemica* 1:10-16.
- Bruder, S. P., N. S. Ricalton, R. E. Boynton, T. J. Connolly, N. Jaiswal, J. Zaia and F. P. Barry. 1998. Mesenchymal stem cell surface antigen SB-10 corresponds to activated leukocyte cell adhesion molecule and is involved in osteogenic differentiation. *J. Bone Miner. Res.* 13(4):655-663.
- Burghardt, R. C., J. A. Bowen, G. R. Newton and F. W. Bazer. 1997. Extracellular matrix and the implantation cascade in pigs. *J. Reprod. Fertil. Suppl.* 52:151-164.
- Burghardt, R. C., G. A. Johnson, L. A. Jaeger, H. Ka, J. E. Garlow, T. E. Spencer and F. W. Bazer. 2002. Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. *Cells, Tissues, Organs.* 172(3):202-217.
- Campbell, K. H. S., R. Alberio, I. Choi, P. Fisher, R. D. W. Killy, J. H. Lee and W. Maalouf. 2005. Cloning: Eight years after Dolly. *Reprod. Domest. Anim.* 40(4):256-268.
- Cayrol, R., K. Wosik, J. L. Berard, A. Dodelet-Devillers, I. Ifergan, H. Kebir, A. S. Haqqani, K. Kreymborg, S. Krug, R. Moudjian, A. Bouthillier, B. Becher, N. Arbour, S. David, D. Stanimirovic and A. Prat. 2008. Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system. *Nat. Immunol.* 9(2):137-145.
- Chae, J. I., Y. K. Cho, S. K. Cho, J. H. Kim, Y. M. Han, D. B. Koo and K. K. Lee. 2008. Proteomic analysis of pancreas derived



- from adult cloned pig. *Biochem. Biophys. Res. Commun.* 366(2):379-387.
- Cortes, F., C. Debacker, B. Peault and M. C. Labastie. 1999. Differential expression of KDR/VEGFR-2 and CD34 during mesoderm development of the early human embryo. *Mech. Dev.* 83:161-164.
- Dantzer, V. 1985. Electron microscopy of the initial stages of placentation in the pig. *Anat. Embryol. (Berl.)* 172(3):281-293.
- Erikson, D. W., R. C. Burghardt, K. J. Bayless and G. A. Johnson. 2009. Secreted phosphoprotein 1 (SPP1, osteopontin) binds to integrin alpha v beta 6 on porcine trophoblast cells and integrin alpha v beta 3 on uterine luminal epithelial cells, and promotes trophoblast cell adhesion and migration. *Biol. Reprod.* 81(5):814-825.
- Farin, P. W., J. A. Piedrahita and C. E. Farin. 2006. Errors in development of fetuses and placentas from *in vitro*-produced bovine embryos. *Theriogenology* 65(1):178-191.
- Fernando, S. C., F. Z. Najjar, X. Guo, L. Zhou, Y. Fu, R. D. Geisert, B. A. Roe and U. DeSilva. 2007. Porcine kallikrein gene family: genomic structure, mapping, and differential expression analysis. *Genomics* 89(3):429-438.
- Fujiwara, H., K. Tatsumi, K. Kosaka, Y. Sato, T. Higuchi, S. Yoshioka, M. Maeda, M. Ueda and S. Fujii. 2003. Human blastocysts and endometrial epithelial cells express activated leukocyte cell adhesion molecule (ALCAM/CD166). *J. Clin. Endocrinol. Metab.* 88(7):3437-3443.
- Geisert, R. D. and J. V. Yelich. 1997. Regulation of conceptus development and attachment in pigs. *J. Reprod. Fertil. Suppl.* 52:133-149.
- Gessert, S., D. Maurus, T. Brade, P. Walther, P. Pandur and M. Kuhl. 2008. DM-GRASP/ALCAM/CD166 is required for cardiac morphogenesis and maintenance of cardiac identity in first heart field derived cells. *Dev. Biol.* 321(1):150-161.
- Hagen, D. R., R. S. Prather, M. M. Sims and N. L. First. 1991. Development of one-cell porcine embryos to the blastocyst stage in simple media. *J. Anim. Sci.* 69(3):1147-1150.
- Hernandez-Gonzalez, I., I. Gonzalez-Robayna, M. Shimada, C. M. Wayne, S. A. Ochsner, L. White and J. S. Richards. 2006. Gene expression profiles of cumulus cell oocyte complexes during ovulation reveal cumulus cells express neuronal and immune-related genes: does this expand their role in the ovulation process? *Mol. Endocrinol.* 20(6):1300-1321.
- Humpherys, D., K. Eggen, H. Akutsu, K. Hochedlinger, W. M. III Rideout, D. Biniszkiwicz, R. Yanagimachi and R. Jaenisch. 2001. Epigenetic instability in ES cells and cloned mice. *Science* 293(5527):95-97.
- Hyun, S. H., G. S. Lee, D. Y. Kim, H. S. Kim, S. H. Lee, S. Kim, E. S. Lee, J. M. Lim, S. K. Kang, B. C. Lee and W. S. Hwang. 2003. Effect of maturation media and oocytes derived from sows or gilts on the development of cloned pig embryos. *Theriogenology* 59(7):1641-1649.
- Ikeda, K. and T. Quertermous. 2004. Molecular isolation and characterization of a soluble isoform of activated leukocyte cell adhesion molecule that modulates endothelial cell function. *J. Biol. Chem.* 279(53):55315-55323.
- Jezierska, A., W. Matysiak and T. Motyl. 2006. ALCAM/CD166 protects breast cancer cells against apoptosis and autophagy. *Med. Sci. Monit.* 12(8):BR263-273.
- Ka, H., L. A. Jaeger, G. A. Johnson, T. E. Spencer and F. W. Bazer. 2001. Keratinocyte growth factor is up-regulated by estrogen in the porcine uterine endometrium and functions in trophoblast cell proliferation and differentiation. *Endocrinology* 142(6):2303-2310.
- Ka, H., H. Seo, M. Kim, S. Moon, H. Kim and C. K. Lee. 2008. Gene expression profiling of the uterus with embryos cloned by somatic cell nuclear transfer on day 30 of pregnancy. *Anim. Reprod. Sci.* 108:79-91.
- Ka, H., T. E. Spencer, G. A. Johnson and F. W. Bazer. 2000. Keratinocyte growth factor: expression by endometrial epithelia of the porcine uterus. *Biol. Reprod.* 62(6):1772-1778.
- Keefer, C. L. 2008. Lessons learned from nuclear transfer (cloning). *Theriogenology* 69(1):48-54.
- Kim, M., H. Seo, Y. Choi, W. Hwang, C. K. Lee and H. Ka. 2009. Aberrant expression of retinol-binding protein, osteopontin and fibroblast growth factor 7 in the porcine uterine endometrium of pregnant recipients carrying embryos produced by somatic cell nuclear transfer. *Anim. Reprod. Sci.* 112:172-181.
- Konno, A., J. S. Ahn, H. Kitamura, M. J. Hamilton, J. A. Gebe, A. Aruffo and W. C. Davis. 2001. Tissue distribution of CD6 and CD6 ligand in cattle: expression of the CD6 ligand (CD166) in the autonomic nervous system of cattle and the human. *J. Leukoc. Biol.* 69(6):944-950.
- Lefevre, F., F. Martinat-Butte, M. Guillomot, K. Zouari, B. Charley and C. La Bonnardiere. 1990. Interferon-gamma gene and protein are spontaneously expressed by the porcine trophoblast early in gestation. *Eur. J. Immunol.* 20(11):2485-2490.
- Levesque, M. C., C. S. Heinly, L. P. Whichard and D. D. Patel. 1998. Cytokine-regulated expression of activated leukocyte cell adhesion molecule (CD166) on monocyte-lineage cells and in rheumatoid arthritis synovium. *Arthritis Rheum.* 41(12):2221-2229.
- Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4):402-408.
- Masedunskas, A., J. A. King, F. Tan, R. Cochran, T. Stevens, D. Sviridov and S. F. Ofori-Acquah. 2006. Activated leukocyte cell adhesion molecule is a component of the endothelial junction involved in transendothelial monocyte migration. *FEBS Lett.* 580 (11):2637-2745.
- Ohneda, O., K. Ohneda, F. Arai, J. Lee, T. Miyamoto, Y. Fukushima, D. Dowbenko, L. A. Lasky and T. Suda. 2001. ALCAM (CD166): its role in hematopoietic and endothelial development. *Blood* 98(7):2134-2142.
- Patel, D. D., S. F. Wee, L. P. Whichard, M. A. Bowen, J. M. Pesando, A. Aruffo and B. F. Haynes. 1995. Identification and characterization of a 100-kD ligand for CD6 on human thymic epithelial cells. *J. Exp. Med.* 181(4):1563-1568.
- Petters, R. M. and K. D. Wells. 1993. Culture of pig embryos. *J. Reprod. Fertil. Suppl.* 48:61-73.
- Pope, W. 1994. Embryonic mortality in swine. In *Embryonic mortality in domestic species* (Ed. M. T. Zavy and R. D. Geisert) pp. 53-78. CRC Press, Florida, USA.
- Ross, J. W., J. R. Malayer, J. W. Ritchey and R. D. Geisert. 2003. Characterization of the interleukin-1beta system during porcine trophoblastic elongation and early placental attachment. *Biol. Reprod.* 69(4):1251-1259.

- Singer, N. G., R. Mitra, F. Lialios, B. C. Richardson, R. M. Marks, J. M. Pesando, D. A. Fox and B. J. Nickoloff. 1997. CD6 dependent interactions of T cells and keratinocytes: functional evidence for a second CD6 ligand on gamma-interferon activated keratinocytes. *Immunol. Lett.* 58(1):9-14.
- Song, G., D. W. Bailey, K. A. Dunlap, R. C. Burghardt, T. E. Spencer, F. W. Bazer and G. A. Johnson. 2010. Cathepsin B, cathepsin L, and cystatin C in the porcine uterus and placenta: potential roles in endometrial/placental remodeling and in fluid-phase transport of proteins secreted by uterine epithelia across placental areolae. *Biol. Reprod.* 82(5):854-864.
- Stephan, J. P., L. Bald, P. E. Roberts, J. Lee, Q. Gu and J. P. Mather. 1999. Distribution and function of the adhesion molecule BEN during rat development. *Dev. Biol.* 212(2):264-77.
- Uchida, N., Z. Yang, J. Combs, O. Pourquie, M. Nguyen, R. Ramanathan, J. Fu, A. Welply, S. Chen, G. Weddell, A. K. Sharma, K. R. Leiby, D. Karagogeos, B. Hill, L. Humeau, W. B. Stallcup, R. Hoffman, A. S. Tsukamoto, D. P. Gearing and B. Peault. 1997. The characterization, molecular cloning, and expression of a novel hematopoietic cell antigen from CD34+ human bone marrow cells. *Blood* 89(8):2706-2716.