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Change of uterine histotroph proteins during follicular and luteal phase in pigs

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ABSTRACT

The aim of this study was to examine protein expression patterns of uterine histotroph (UH) during the follicular phase (FP) and luteal phase (LP) in pigs. Forty-nine common proteins were identified from FP and LP samples; five were significantly down-regulated (>1.5-fold), while 15 were significantly up-regulated (>1.5-fold) in LPUH compared with FPUH ($P < 0.05$). The 20 differentially-expressed proteins are involved in cell proliferation, cell responses, translation, transport, and metabolism and their molecular functions include nucleic acid binding, oxygen activity, enzymatic activity, growth activity, iron binding, and redox binding. Protein expression of vascular endothelial growth factor D (VEGFD), coatomer subunit gamma-2 (G2COP), collagen alpha 4 chain (COL4), cysteine rich protein 2 (CRP2), myoglobin (MYG), and galactoside 3-L-fucosyltransferase 4 (FUT4) was analyzed by Western blotting. These proteins were significantly higher in LPUH compared to FPUH ($P < 0.05$). These data expand our understanding of changes in the intrauterine environment during the pre-implantation period in pigs.

1. Introduction

In mammals, the endometrium undergoes a repetitive cycle of proliferation, differentiation and repair to prepare the uterus for the establishment and maintenance of pregnancy. The follicular phase (FP) and luteal phase (LP) of the estrous cycle are important in preparing for ovulation and implantation, respectively. The uterine glandular epithelium is also higher in LP compared to FP (Gray et al., 2001; Hettinger et al., 2001). Angiogenesis (Kaczmarek et al., 2010), apoptosis (Ziecik et al., 2011) and extracellular matrix (ECM) remodeling (Diao et al., 2011) are important processes involved in the development of the uterine glandular epithelium in mammals.

Uterine glands develop to facilitate implantation of the embryo during the estrous cycle in sheep (Bartol et al., 1988; Taylor et al., 2000), pigs (Spencer et al., 1993; Tarleton et al., 1998), rats (Branham and Sheehan, 1995), and mice (Bigsby and Cunha, 1985). Secretion of substances synthesized or transported in the uterine glands play an essential role in the survival and development of the embryo, or conceptus.

All mammalian uteri contain glandular and luminal epithelium in the endometrium, that secret 'histotroph', a complex mixture of hormones, enzymes, growth factors, cytokines, proteins, adhesion factors, nutrients, and other substances that play roles in conceptus nourishment, implantation and placentation of the conceptus (Ashworth and Bazer, 1989; Gray et al., 2000). Thus, uterine histotroph (UH) provide an important source for porcine concep-

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tus growth (Tarraf and Knight, 1995). Protein contents of intrauterine luminal histoph are composed of serum and unique substances from the uterus (Ringler, 1961; Kulangara, 1972; Beier, 1974). Therefore, proteins of the UH are likely to play essential roles in the endometrium environment and embryonic development.

In this study, we used two-dimensional electrophoresis (2-DE) to examine protein expression profiles of UH derived from different phases of the estrous cycle before implantation in pigs. We also determined whether the biological processes and molecular functions of the proteins are associated with physiological and morphological functions in the intrauterine environment.

2. Materials and methods

2.1. Uterine histoph preparation

All procedures involving animals were approved by the Kangwon National University Institutional Animal Care and Use Committee (KIACUC-09-0139). Estrous induction and synchronization were performed in 14 crossbred gilts (Yorkshire × Landrace) weighing 140–160 kg by hormonal treatment (Jalali et al., 2015). Gilts were randomly classified into two groups of 7 gilts at detection of estrous (Day 0). The gilts were slaughtered at a nearby abattoir (Pocheon farm, Korea) either on Day 10 (LP) or Day 18 (FP) of the estrous cycle, and the experimental gilts are 7.1 ± 0.4 months. Uteri were obtained from FP (n = 7) and LP (n = 7) sows, and transported to the laboratory within 2 h at 4 °C. Uteri were flushed with PBS for collection of UH, then the UH were centrifuged at 4500*g* for 5 min at 4 °C, after which, the supernatants were filtered with a 0.2 μm syringe filter (Sartorius, Gottingen, Germany) and stored at −80 °C until use.

2.2. Protein sample preparation

UH samples were lyophilized, and re-suspended in mammalian protein extraction buffer (Thermo, Waltham, MA, USA). Samples were rotated for 1 h at room temperature, and centrifuged at 4 °C (12,000*g*, 10 min). The supernatant was then transferred into a new microcentrifuge tube. Protein concentration was determined by the Bradford method (Bradford, 1976). Components of the extracted protein that interfered with substances such as detergents, slats, lipids, phenolics and nucleic acids were removed using the 2-D Clean-Up Kit (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions, and 750 μg protein was dissolved in 300 μL rehydration solution (GE Healthcare) for 1 h at room temperature.

2.3. Two-dimensional gel electrophoresis

Proteins in rehydration buffer were incubated with an 18 cm immobilized pH 3–11 nonlinear gradient dry strip (GE Healthcare) for 16 h at room temperature. As described previously (Lee et al., 2011), isoelectric focusing (IEF) was performed for protein separation. IEF was performed at 250 V for 2 h, 8000 V for 3 h, and up to a total of 60,000 V. Strips were then equilibrated for 15 min in 5 mL equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol (v/v), and 2% sodium dodecyl sulfate (w/v) containing 80 μg dithiothreitol (DTT; Sigma, St Louis, MO, USA), followed by an additional incubation for 15 min in 5 mL equilibration buffer containing 100 μg iodoacetamide. Separation in the second dimension was accomplished using an 8% SDS polyacrylamide gel in a Protean II xi 2-D cell (Bio-Rad, Hercules, CA, USA) at 50 mA until the bromophenol blue reached the bottom of the gel. Gels were stained in a solution of 0.1% Coomassie Brilliant Blue R-250 (Sigma) comprised of 45% methanol, 10% acetic acid and 45% water. Gels were then scanned using an image scanner (Amersham) and analyzed with Phoretix Expression software (Nonlinear Dynamics, Cambridge, CS, UK) and each phase UH protein spots intensities were normalized to FPUH protein spots intensities for calculation of relative spot intensity.

2.4. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS)

Spots were extracted from the gel and washed three times for 15 min each in 50% acetonitrile (ACN; Sigma) containing 25 mM NH_4 bicarbonate. After washing, samples were incubated with 50% ACN containing 10 mM NH_4 bicarbonate for 15 min, and then incubated with 100% ACN for 5 min. Finally, ACN in samples was removed using a speed vacuum. Then, samples were incubated with cold sequencing-grade modified trypsin (Promega, Madison, WI, USA) at 37 °C for 20 h, followed by a 50 min incubation with 50% ACN containing 5% trifluoroacetic acid (TFA) at room temperature. The supernatants were dried for peptide extraction using a speed vacuum, and then diluted with 50% ACN containing 5% TFA. Samples were desalted using a Zip-Tip C18 (Millipore, Milford, MA, USA). Plating was performed using a 4-hydroxy-α-cyano-cinnamic acid matrix solution (Sigma) on a MALDI-TOF/MS plate. Peptides were analyzed using an Ultraflex-TOF/TOF spectrometer (Bruker Daltonics, Hamburg, Germany) and MASCOT 2.0 software (Matrix science, Boston, MA, USA). The NCBI non-redundant protein database (http://www.ncbi.nlm.nih.gov/) was used to search for protein sequences.

2.5. Western blotting

UH proteins (50 μg/20 μL) were separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 1 h, transferred to a polyvinylidene difluoride (PVDF) membrane for 90 min at 90 V, and incubated in blocking solution (5% skim milk in Tris-buffered saline/0.5% Tween-20; TBS-T) for 1 h at room temperature. All primary antibodies and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were incubated with goat polyclonal primary antibodies raised against IgG anti-coatomer subunit gamma-2 (G2COP; sc-23204), cysteine-rich protein 2 (CRP2; sc-167547) and collagen alpha 4 chain (COL4; sc-9302), rabbit polyclonal antibodies targeting IgG anti-vascular endothelial growth factor D (VEGFD; sc-25784), myoglobin (MYG; sc-25607) and galactoside 3-L-fucosyltransferase 4 (FUT4;