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Proteomic analysis of porcine endometrial tissue during peri-implantation period reveals altered protein abundance

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ABSTRACT

In mammals, successful pregnancy depends upon the readiness of uterus for implantation, followed by correct communication between the endometrium and the developing conceptus. The objective of this study was to elucidate changes in protein abundance associated with progression of estrous cycle and pregnancy from Day 9 to Day 12. We analyzed porcine endometrial tissue lysates by 2D-DIGE. Abundance of several proteins was altered depending upon the pregnancy status of animals. MALDI-TOF/TOF was used to identify a number of these proteins. Endometrial proteins that increased from Day 9 to Day 12 of cycle included annexin A4, beta-actin, apolipoprotein, ceruloplasmin and afamin. Changes in protein abundances associated with conceptus secreted factors, including haptoglobin, prolyl-4-hydroxylase, aldose-reductase and transthyretin, were also observed. Functional analysis revealed that endometrial proteins with altered abundance on Day 12 irrespective of the reproductive status were related to growth and remodeling, acute phase response and free radical scavenging, whereas transport and small molecule biochemistry were the functions activated in the pregnant endometrium as compared to the cyclic endometrium. These data provide information on dynamic physiological processes associated with uterine endometrial function of the cyclic and pregnant endometrium during period of maternal recognition of pregnancy in pigs and may potentially demonstrate a protein profile associated with successful pregnancy.

Biological significance
In pigs, the fertility rates are generally very high but the early embryonic loss that occurs during the second and third weeks of gestation critically affects the potential litter size. Temporal changes that take place in the uterine environment during the period of early pregnancy in pigs and a cross-talk between the uterus and the embryo play an important role in embryonic survival and successful pregnancy. A better understanding of the molecular changes associated with these processes will pave way for understanding of endometrial functions and help towards increasing embryo survival. In this study, we present a 2D-DIGE based analysis of changes in porcine endometrial proteome that are associated with progression of cycle and progression of pregnancy. The network analysis of the results clearly revealed the pathways that are involved in rendering the endometrium receptive to the presence of embryo and also the changes that are result of molecular communication between the endometrium and the conceptuses. This comprehensive identification of proteomic changes in the porcine endometrium could be a foundation for targeted studies of proteins and pathways potentially involved in abnormal endometrial receptivity, placentation and embryo loss.

1. Introduction

The establishment of pregnancy requires the participation of a receptive endometrium and the development of the embryo to the implantation competent stage [1,2]. In mammalian species, including pigs, the endometrium undergoes a transformation in response to the physiological changes triggered by ovarian hormones in different stages of the cycle to prepare for embryo attachment and implantation [3]. These transformations involve changes to the endometrial structure and spatiotemporal alterations in molecular profiles. The endometrium also secretes a wide array of growth factors, proteins and cytokines, which constitute the histotroph, an important source of energy and nutrition to a growing embryo [4,5]. In both cycling and pregnant animals, similar molecular changes occur in the endometrium up to the initiation of conceptus elongation [6], suggesting that the changes responsible for the formation of a receptive endometrium are initially under maternal control. The endometrial receptivity triggered by ovarian steroids is later modified by the molecular signaling between the embryo and the endometrium. In pigs, this signaling between the embryo and

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endometrium starts around Days 11 to 12 of pregnancy when there is a surge of estrogens secreted by the conceptuses in the uterine lumen [7]. This period is also known as the maternal recognition of pregnancy in pigs. It has been hypothesized that an increase in estrogen concentration stimulates the secretion of endometrial proteins, promoting trophoblast growth, which are critical for implantation [8].

Using the candidate gene approach, many studies have evaluated gene and protein expressions in the porcine endometrium to elucidate the molecular mechanisms responsible for the establishment of pregnancy in pigs [3,9,10]. These efforts, along with global gene expression studies, have tremendously advanced our understanding of porcine pregnancy and endometrial biology. In recent years, while many global gene expression studies in the porcine endometrium have been conducted on differential gene expression during pregnancy [11–14], there are very few reports regarding global changes in porcine endometrial proteome during pregnancy [6,15]. Kayser et al. [6] reported changes in uterine fluid proteome during early porcine pregnancy and some proteins reported by them are common to this study. However, as uterine fluid does not contain high abundance cellular proteins, it is expected to have a less complex proteome than that of endometrial tissue. Given the complexity of the endometrium, the uterine fluid and endometrial proteome would not be expected to be quantitatively or qualitatively identical, though common proteins could be identified.

In our study, 2D-DIGE was used to construct a 2D map of the cycling and pregnant porcine endometrium to identify the proteins that are differentially expressed with the progression of the estrous cycle and pregnancy. We observed both estrous cycle and pregnancy-dependent changes and also identified a number of proteins that have not previously been identified in the porcine endometrium.

2. Materials and methods

2.1. Animals

All procedures involving the use of animals were approved by the Animal Ethics Committee, University of Warmia and Mazury in Olsztyn, Poland, and were conducted in accordance with the national guidelines for agricultural animal care. Estrous induction and synchronization were achieved in 16 crossbred gilts (*Sus scrofa domesticus*) weighing ~100 kg by hormonal treatment with administration of an intramuscular injection of 750 IU of equine chorionic gonadotropin followed by 500 IU of human chorionic gonadotropin (hCG), 72 h later. Following induced estrous, gilts were randomly divided into two groups of 8 gilts each which received treatments of either a uterine infusion of sterilized PBS (cyclic group), or two artificial inseminations (AI) with 2.5×10^9 spermatozoa within an interval of 12 h (pregnant group). AI was carried out 24 h after hCG injection. The gilts were slaughtered at a local abattoir either on Day 9 ($n = 4$) or Day 12 of the estrous cycle ($n = 4$) or on Day 9 ($n = 4$) or Day 12 ($n = 4$) of pregnancy. Uteri were collected and flushed with PBS, then the endometrium was separated from the myometrium by opening the uterus longitudinally on the antimesometrial surface and snap frozen in liquid nitrogen and stored at −80 °C until further analysis. Pregnancy was confirmed by the presence of conceptuses in the uterus.

2.2. Sample preparation

The tissue samples were homogenized using a ceramic mortar and pestle, precooled with liquid nitrogen for at least 1 min. Homogenized frozen tissue (~30 mg) was directly transferred into DIGE lysis buffer pH 8.5 (30 mM Tris–HCl, 7 M urea, 2 M thiourea and 4% w/v CHAPS). Lysates were sonicated for 3 min with a Sonics Vibra-Cell VCX 130 and centrifuged in Beckman Ultracentrifuge J2-HS at 20,000 rpm for 30 min at 4 °C. Protein concentrations of lysates were determined by Bradford assays [16].

2.3. Fluorescence labeling of samples with CyDyes

The endometrial protein samples (50 μg) from individual cyclic and pregnant animals were dissolved in labeling buffer (7 M urea, 2 M thiourea, 2% w/v CHAPS and 30 mM Tris; final pH of 8.0) and labeled with CyDye DIGE Fluor minimal dyes (GE Healthcare, Uppsala, Sweden) reconstituted in fresh 99.8% anhydrous dimethylformamide (DMF) at a concentration of 400 pmol of dye/50 μg of protein. Cy3 and Cy5 were used to label individual samples from cyclic or pregnant animals, applying a cross-labeling scheme (Fig. S1). Equal amounts (25 μg) of individual cyclic and pregnant endometrial protein lysates were mixed as an internal standard and labeled with Cy2. Labeling reaction was performed in the dark on ice for 30 min. The reaction was stopped by adding 1 μl of 10 mM lysine followed by incubation in the dark on ice for 10 min. Equal protein amounts (50 μg) of Cy2, Cy3 and Cy5-labeled samples were mixed and added to an equal volume of sample buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% DTT, and 2% v/v Pharmalyte pH 4–7 or 6–11), and loaded on to pre-rehydrated IPG strips.

2.4. Two dimensional PAGE

Iso electric focusing (IEF) was performed using Ettan IPGphor IEF System I and 18 cm Immobiline DryStrips pH 4–7, or pH 6–11 (both GE Healthcare Bio-Sciences). DryStrips were pre-rehydrated for 9 h (passive rehydration) in rehydration buffer (7 M urea, 2 M thiourea 2% w/v CHAPS, 10 mM DTT, 1% v/v Pharmalyte pH 4–7 or pH 6–11) followed by anodic cup loading, and the IPG strips were focused for a total of 37.15 kVh. The focused IPG strips were stored in long tubes at −80 °C. Prior to SDS PAGE electrophoresis each strip was equilibrated in 10 ml equilibration buffer (50 mM Tris–HCl pH 6.8, 6 M urea, 30% v/v glycerin, and 2% w/v SDS) containing 1% w/v DTT and a trace of bromophenol blue for 15 min at room temperature. A second equilibration step was carried out for 15 min in 10 ml of same equilibration buffer that contained 2.5% w/v iodoacetamide instead of DTT. For the second dimension analysis, samples were separated on 12.5% DIGE gels using the DIGE buffer kit (GE Healthcare) in the Ettan DALTsix vertical electrophoresis system (GE Healthcare). IEF gel strips were placed onto the second dimension 12.5% SDS-PAGE gels and run at 20 °C at a constant current of 10 mA/gel for 40 min and then 50 mA/gel until the dye reached the bottom of the gel.

2.5. Image acquisition and quantitative analysis

The gels were scanned with a Typhoon 9400 variable mode imager (GE Healthcare) using the manufacturer suggested parameters. Images were analyzed with the DeCyder Differential in Gel Analysis version 5.02 software (GE Healthcare) to identify spot fluorescence intensities. The DeCyder biological variation analysis module was used to detect protein spots and simultaneously match all twelve protein spot maps from four gels in one group. The comparisons were made between 1) Day 9 of cycle and Day 12 of cycle (9 DC and 12 DC, respectively) and 2) Day 9 of pregnancy and Day 12 of pregnancy (9 DP and 12 DP, respectively). Proteins were considered to be differentially expressed if their spots had a minimum of 1.8-fold change in their relative abundance with $p < 0.05$ by Student's *t*-test compared to any other group. In the present study, each *t*-test compared two protein expression values and not multiple comparisons. False discovery rate correction was applied when the *t*-test was performed. Spots of interest identified through these analyses were verified to have 3-D profile characteristics of a protein spot, and features detected from non-protein sources (particles and backgrounds) were filtered out. Only spots that were successfully matched on >80% of the gel images were considered for further analyses. Spots selected for additional analysis were picked from separate preparative gels loaded with 900 μg of proteins lysates from cyclic or pregnant animals and stained with CBB.