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Global characterization of porcine intrauterine proteins during early pregnancy

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Abstract

Total protein secreted in the intrauterine lumen increases between day 10 and 13 post-estrus in both cyclic and pregnant gilts. The objective of this experiment was to identify those intrauterine proteins whose secretion changes during this time period. Sixteen mature gilts were either mated (day 0) or remained cyclic and were slaughtered at either day 10 or day 13 ($n = 4$ per status by day). At slaughter, each uterine horn was flushed with 20 ml Minimal Essential Medium. Flushings were dialyzed extensively against distilled water. A 0.5 ml aliquot of each was lyophilized, subjected to two-dimensional PAGE, and protein spots were identified following Coomassie staining of each gel. Densitometry was used to compare relative amounts of each spot. After statistical analysis, spots that differed due to either day, status, or day by status interaction were excised and digested in-gel with trypsin. The resulting peptides were analyzed by tandem mass spectrometry (MS/MS). Using MS/MS data, protein identification for each spot was attempted. There were 280 matching spots, of which 132 were significantly ($P < 0.05$ or 0.01) affected by pregnancy status, day, or the day by status interaction. Most (73%) spots increased from day 10 to day 13 with no effect of pregnancy. Several spots were identified as proteases or their inhibitors. Others potentially modify glycolipids and/or glycoproteins. These results indicate that the concentrations of many proteins within the intrauterine environment during early pregnancy are independent of the conceptus and could play roles in regulating the endometrial or conceptus glycocalyx.

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Introduction

Uterine secretions play an important role in orchestrating the synchrony between uterine receptivity and early development of the conceptus including conceptus remodeling, adhesion, implantation and placentation (Burghardt *et al.* 2002). During maternal recognition of pregnancy (day 10–13), the pig conceptus undergoes a morphological transformation from a spherical blastocyst to an elongated filamentous conceptus (Geisert *et al.* 1982a). Pig trophoblast can penetrate non-uterine epithelia and develop at ectopic sites (Samuel & Perry 1972). However, *in utero*, the epithelial glycocalyx, stimulated by progesterone early during the estrous cycle or pregnancy, remains abundant throughout conceptus attachment and pregnancy, possibly providing a barrier between the proteolytic trophoblast and the uterine epithelial cells (Geisert *et al.* 1995, Ferrell *et al.* 2003). Protease inhibitors secreted by the uterus protect the uterine epithelium from proteases and may function to promote the initial

attachment of the embryo through a protein–integrin receptor (Burghardt *et al.* 2002).

Estrogen secretion by the conceptus occurs simultaneously with trophoblast elongation and is thought to function as the signal for maternal recognition of pregnancy (Bazer & Thatcher 1977, Geisert *et al.* 1982a,b). In addition, it has been hypothesized that increasing estrogen concentrations in the uterine lumen stimulate the secretion of endometrial proteins that promote trophoblast expansion (Geisert *et al.* 1982b,c, 1995). However, the concentration of total protein in the uterus dramatically increased in pregnant and cyclic gilts between day 10 and 13 post-ovulation (Vallet *et al.* 1996, 1998a) suggesting very limited effects of the conceptus on this process. The absence of conceptus during the estrous cycle suggests that uterine secretion of many proteins during this period may be under maternal control. Several highly abundant proteins in uterine fluid have been detected and described including: uteroferrin (Roberts & Bazer 1988), retinol-binding protein (RBP) (Harney *et al.* 1990, Stallings-Mann *et al.* 1993), plasmin

trypsin inhibitor (Fazleabas *et al.* 1983), antileukoprotease (Simmen *et al.* 1991) and folate-binding protein (Vallet *et al.* 1998b). However, many proteins found in the uterine lumen during the period of maternal recognition of pregnancy have not been identified.

Global protein expression changes during most developmental stages in animals. This can be attributed to the dynamic nature of cellular processes involved in these stages, especially post-translational modifications that alter the function of proteins. Obtaining information on proteins has been limited by the ability to measure and identify proteins with high throughput. High resolution two-dimensional PAGE (2D-PAGE) described by O'Farrell (1975) is commonly used to separate proteins from a complex biological mixture. Following staining, 2D-PAGE provides a global overview of proteins expressed in or secreted by certain cells or tissue types due to a given physiological state (Shevchenko *et al.* 1996a). The level of protein expression is reflected in the staining intensity of the protein spot. The combination of this technique with protein identification using mass spectrometry (Pandey & Mann 2000, Standing 2003) is capable of identifying and characterizing multiple proteins simultaneously that may provide clues to the functional networks during a biological process (Shankar *et al.* 2005).

Mass spectrometry provides high-throughput, accurate mass measurements, and reasonable certainty of identification for a large number of unknown proteins separated by 2D-PAGE (Shevchenko *et al.* 1996a). However, protein identification based on mass mapping works best for those species in which relatively complete genome sequence information is available. Matching candidate peptides to database sequences with weaker homology, for species like the pig, is facilitated by implementing a *de novo* interpretation of tandem mass spectrometry (MS/MS) spectra followed by a homology-tolerant search of related species (Clauser *et al.* 1999, Taylor & Johnson 2001, Mackey *et al.* 2002). We have previously defined parameters for identifying proteins from pigs by homology-tolerant searching (Kayser *et al.* 2004) and employed those techniques in this study.

Thus, we combined 2D-PAGE and MS/MS to identify uterine luminal proteins that change between day 10 and 13 in order to provide clues to the various physiological processes occurring during this period. Our objectives were to (i) characterize the changes in the uterine protein profiles between cyclic and pregnant gilts on day 10 and 13 post-estrus, and (ii) identify those proteins that differed significantly during this period of pregnancy and the estrous cycle.

Materials and Methods

All experiments were performed according to Federation of Animal Science Society guidelines for the use of agricultural animals in research, and each experiment was reviewed and approved by the Meat Animal Research

Center's (MARC) Institutional Animal Care and Use Committee. Intact white crossbred gilts were checked once daily for estrous behavior through one complete cycle before being assigned to treatments. On the first day of standing estrus (day 0), gilts were randomly assigned to be either mated ($n = 8$) by artificial insemination or remain cyclic ($n = 8$). Four gilts randomly assigned within each group ($n = 4/\text{status}$) were slaughtered on day 10 and the remaining gilts were slaughtered on day 13 post-estrus.

Porcine intrauterine proteins were collected by flushing each uterine horn with 20 ml Minimal Essential Medium and stored at -80°C . Uterine flushings were dialyzed extensively against distilled water (three changes) to remove salts and 0.5 ml aliquots of each flushing were lyophilized. Proteins were solubilized in 5 mM K_2CO_3 , 9.6 M urea, 50 mM dithiothreitol. Proteins were isoelectric focused in 11 cm, linear pH 3–10 tube gels (4% acrylamide, 0.7% crosslinker). Next, proteins were separated in the second dimension using a 10% acrylamide gel and a 4.5% acrylamide stacking gel as previously described (Roberts *et al.* 1984). Gels were stained with Coomassie blue to visualize proteins within each gel.

Individual Coomassie-stained 2D-PAGE gels were recorded as 8 bit TIFF images at 300 dpi using a large bed format image scanner (ImageScanner II; Amersham Biosciences Corp., Piscataway, NJ, USA) and Phoretix PowerScan software (version 3.01, Nonlinear Dynamics, Newcastle upon Tyne, UK). Detection of each spot and spot matching between gels was performed manually using Phoretix 2D Advance software (version 6.01, Nonlinear Dynamics). In our experience, this resulted in the most uniform, repeatable spot detection and matching using this software. Individual gels were matched to a reference gel selected from one of the day 13 pregnant gilts which displayed the most complete set of protein spots. To validate the matching of spots between gels, ten randomly selected protein spots were excised from separate 2D-PAGE gels representing day 10 cyclic and day 13 pregnant (pooled within treatment) intrauterine proteins. These two groups were selected because they showed the greatest disparity between secreted proteins, which made protein spot matching more difficult. Successful spot matches were determined from protein identification using mass spectrometry data as described below.

Protein spots that differed between pregnancy status, day, or status by day interaction were excised from gels followed by in-gel digestion with 20 ng/ μl trypsin (Promega, Madison, WI, USA) according to published procedures with some modifications (Shevchenko *et al.* 1996b). Peptides were extracted from the gel and injected onto a C18 reversed phase LC column (LC-Packings; Dionex Co., San Francisco, CA, USA) connected to a mass spectrometer. MS/MS (Q-TOF Ultima, Waters; Micromass UK, Beverly, MA, USA) with electrospray ionization was used to analyze eluting peptides. The system was user-controlled with MassLynx software (version 3.5; Micromass) and data-dependent acquisition was performed

using the following parameters: 1 s survey scan (380–1900 Da) followed by up to three 2.4 s MS/MS acquisitions (60–1900 Da).

Raw data obtained from each MS/MS spectrum were processed using Proteinlynx software (version 3.5; Micro-mass) to generate a list (.pkl file) of masses of precursor peptide ions and their fragments. These data were used to generate a *de novo* amino acid sequence for each doubly charged precursor ion (PEAKS software, version 2.0; Bioinformatics Solutions Inc., ON, Canada). The resulting peptide sequences were queried en masse against the NCBI nr protein database (05/10/04) using MS-Homology (Protein-Prosector; University of San Francisco, CA, USA, <http://prosector.ucsf.edu>). Identification of proteins was considered successful if the matching protein was the highest scoring protein for the homology search and if the score was above a significant empirically derived threshold (protein score >51) as previously described (Kayser *et al.* 2004). Protein identification was attempted on all spots deemed statistically significant for one or more effects from at least two different 2D-PAGE gels. Identified proteins were classified according to either biological or molecular function using an integrative biological annotation analysis (PANDORA, Protein ANnotation Diagram ORiented Analysis, version 3.1; www.pandora.cs.huji.ac.il, Kaplan *et al.* 2003), based on SwissProt (www.expasy.org/swissprot/) keywords. From this, a list of proteins sharing keywords and keywords of its ancestors is generated.

To assess the accuracy of protein identifications by mass spectrometry, immunoblots were used to confirm the identification of selected proteins (Table 1) which displayed multiple isoforms. Uterine flush samples were separated in 12.5% polyacrylamide 2D-PAGE gels as previously described (Vallet *et al.* 1996). Proteins were blotted onto nylon-supported nitrocellulose and then incubated in buffer (50 mM Tris pH 7.6, 0.5 M NaCl and 1% Triton X-100) overnight at 4°C. The blots were then incubated for 2 h at room temperature with a 1:1000 dilution of the specific protein antiserum, washed four times with buffer (20 min incubation per wash), incubated with a 1:10 000 dilution of IgG-horseradish peroxidase antibody conjugate for 2 h at room temperature, and then washed four times with buffer. The blots were then rinsed with 0.9% saline. Specific binding was detected with 3,3'-diaminobenzidine plus nickel

sulfate. Furthermore, the identification of an additional spot identified as secreted folate-binding protein was compared with a 2D-PAGE gel of 10 µg of this protein purified from allantoin fluid (Vallet *et al.* 1998b).

Statistical and data analysis

Log-transformed densitometry data for each spot were analyzed using PROC GLM (SAS Institute, Cary, NC, USA) using a model that included the effects of day (10 or 13), status (cyclic or pregnant), and the day by status interaction. In cases where a spot was absent on a particular gel, the densitometry value for that spot was arbitrarily set to 1 (so that the $\log_{10} = 0$). Many spots were absent on all gels from a particular day by status combination. This decreased the pooled error variance for the overall analysis for that spot, because the error variance for that day by status treatment combination was zero. As a conservative approach to this problem, the pooled error variance for these analyses were recalculated after subtraction of the degrees of freedom resulting from the day by status combinations in which all spots were assigned a value of 1. The F statistics were then recalculated using the new, more conservative error term and degrees of freedom.

Results

Following 2D-PAGE, 280 protein spots were manually detected. Representative 2D-PAGE gels of the intrauterine proteome from day 10 of the estrous cycle and day 13 of pregnancy (Fig. 1) show the disparity between day 10 of the cycle and 13 of pregnancy in detected proteins following Coomassie blue staining. For the majority of spots (148/280), no significant day, pregnancy status, or day by pregnancy interaction (Table 2) was detected. Ninety-six spots increased ($P < 0.01$ or < 0.05) between day 10 and 13, irrespectively of pregnancy status (Fig. 1). In contrast, only four spots were greater on day 10 compared with day 13. Six spots were more intense from pregnant than cyclic gilts regardless of day, whereas only one protein spot appeared to increase in cyclic gilts regardless of day. Four spots increased between day 10 and 13, and also were greater in pregnant vs cyclic gilts. A pregnancy by day interaction was detected for 21 spots, and 14 of these were increased only in pregnant gilts on day 13. Likewise,

Table 1 Proteins and antibodies used for immunoblots.

Protein	Primary antibody	Source	Secondary antibody	Source
Albumin	Rabbit anti-human	Sigma ^a	Anti-rabbit whole molecule peroxidase conjugate	Sigma
Transferrin	Goat anti-human	Sigma	Rabbit anti-goat IgG peroxidase conjugate	Sigma
Alpha-2-macroglobulin	Rabbit anti-human	abcam ^b	Anti-rabbit whole molecule peroxidase conjugate	Sigma
Matrix metalloproteinase-2	Rabbit anti-human	Sigma	Anti-rabbit whole molecule peroxidase conjugate	Sigma
Tissue inhibitor of metalloproteinase-2	Rabbit anti-human	Sigma	Anti-rabbit whole molecule peroxidase conjugate	Sigma
Superoxide dismutase	Mouse monoclonal anti-human	Sigma	Goat anti-mouse IgG peroxidase conjugate	Sigma

^aSigma Chemical Company, St Louis, MO, USA.

^babcam, Cambridge, MA, USA.

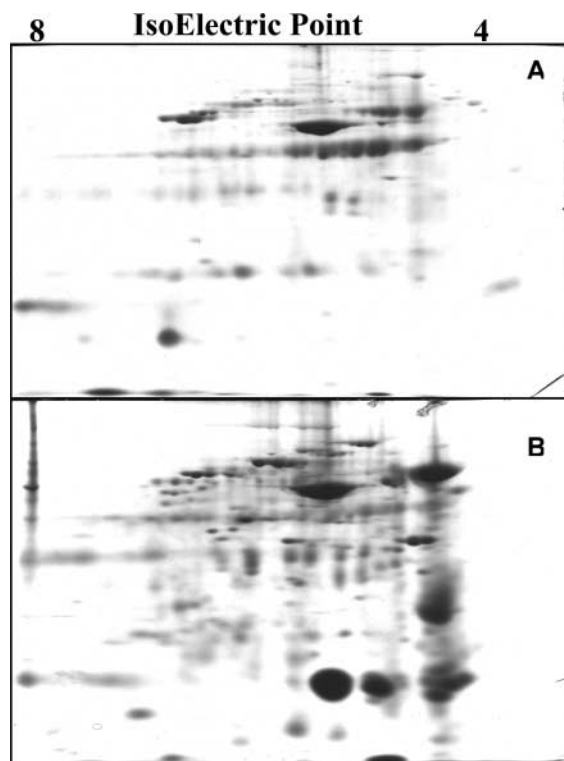


Figure 1 Representative 2-D PAGE gels showing intrauterine proteins isolated on day 10 from cyclic gilts (A), or on day 13 from pregnant gilts (B).

intensity values for the other seven spots increased only on day 10 in cyclic gilts.

A protein map representing the position of 47 proteins positively identified using MS/MS data is shown in Fig. 2. A single protein identification was made for a majority (71.2%) of the spots after repeated MS/MS analysis. However, 37 spots had differing protein identifications between gels, with 29 spots identified as two different proteins and eight spots identified as three different proteins. Our validation of spot matching between day 10 and day 13 gels resulted in nine of ten spots with identical protein identifications. One spot (number 72) was not identified as the

Table 2 Intrauterine proteins that changed significantly due to day, pregnancy status or the day by status interaction^a.

	Number of proteins
Total protein spot matches examined	280
Day 13 > day 10 regardless of status	96
Day 10 > day 13 regardless of status	4
Cyclic > pregnant regardless of day	1
Pregnant > cyclic regardless of day	6
Day 13 > day 10 pregnant (day by status significant)	14
Day 10 > day 13 cyclic (day by status significant)	7
> day 13 and > pregnant (no interaction)	4
Insufficient statistical evidence for change	148

^aLevel of significance determined log-transformed densitometry data ($P \leq 0.05$ or 0.01).

highest scoring protein using MS-Homology from that particular day 10 2D-PAGE. However, meprin-A has been assigned to that spot based on MS/MS data obtained from subsequent gels. Thus, proteins expressed on both day 10 in cyclic gilts and day 13 in pregnant gilts migrated to similar positions within each respective gel regardless of the number of proteins that were expressed. Immunoblots of six proteins confirmed protein identifications and the presence of multiple isoforms (range 2–36) detected for many (40.5% of the total number of significant spots) of the proteins identified. Figure 3 shows a representative immunoblot for albumin. Forty-seven isoforms of albumin were identified using MS/MS data. Immunoblot confirmed the presence and location of 36 albumin isoforms.

Interpretation of the biological relationships among the identified uterine proteins using PANDORA classification of SwissProt keywords are listed in Table 3. The 47 submitted proteins were grouped according to Gene Ontology annotation into one of seven categories based on molecular function or biological processes. Multifunctional proteins were classified according to primary function. The majority of spots ($n = 70$) were classified as serum transport or binding proteins. The largest subgroup of these proteins (54 spots) binds or transports lipids with albumin having the greatest number of isoforms ($n = 47$). Four proteins involved in transport of iron were identified for 13 spots. Twelve proteins were classified as those involved with protein metabolism and modification. Of these, 22 spots represent six proteins that are proteases while 27 spots correspond to seven protease inhibitor proteins. Eleven proteins representing 20 spots function as either carbohydrate (eight proteins) or lipid (three proteins) metabolic proteins. Four proteins (ten spots) function as oxidoreductases to regulate free-radical production. An additional four proteins were identified as immunological proteins. Finally, seven spots were recognized as either structural or cellular proteins.

Discussion

In this study, we demonstrate the dramatic changes that occur in the intrauterine proteome between day 10 and day 13 of the estrous cycle or pregnancy. Using 2D-PAGE to separate individual proteins, we were able to capture the dynamic changes of the functional intrauterine proteome during maternal recognition of pregnancy in the pig. The representative gels in Fig. 1 clearly show the changes in the intrauterine protein profile between day 10 of the estrous cycle and day 13 of pregnancy. We determined that 47% of the protein spots or clusters of proteins changed in expression levels during this period. Significant changes in protein expression ranged from less than 3- to over 1000-fold (spots 83 and 115 respectively). In this study, all gels were stained with Coomassie blue; therefore, the sensitivity of our assay was limited to proteins $> 1 \mu\text{g/ml}$ (500 ng/spot). Our ability to detect

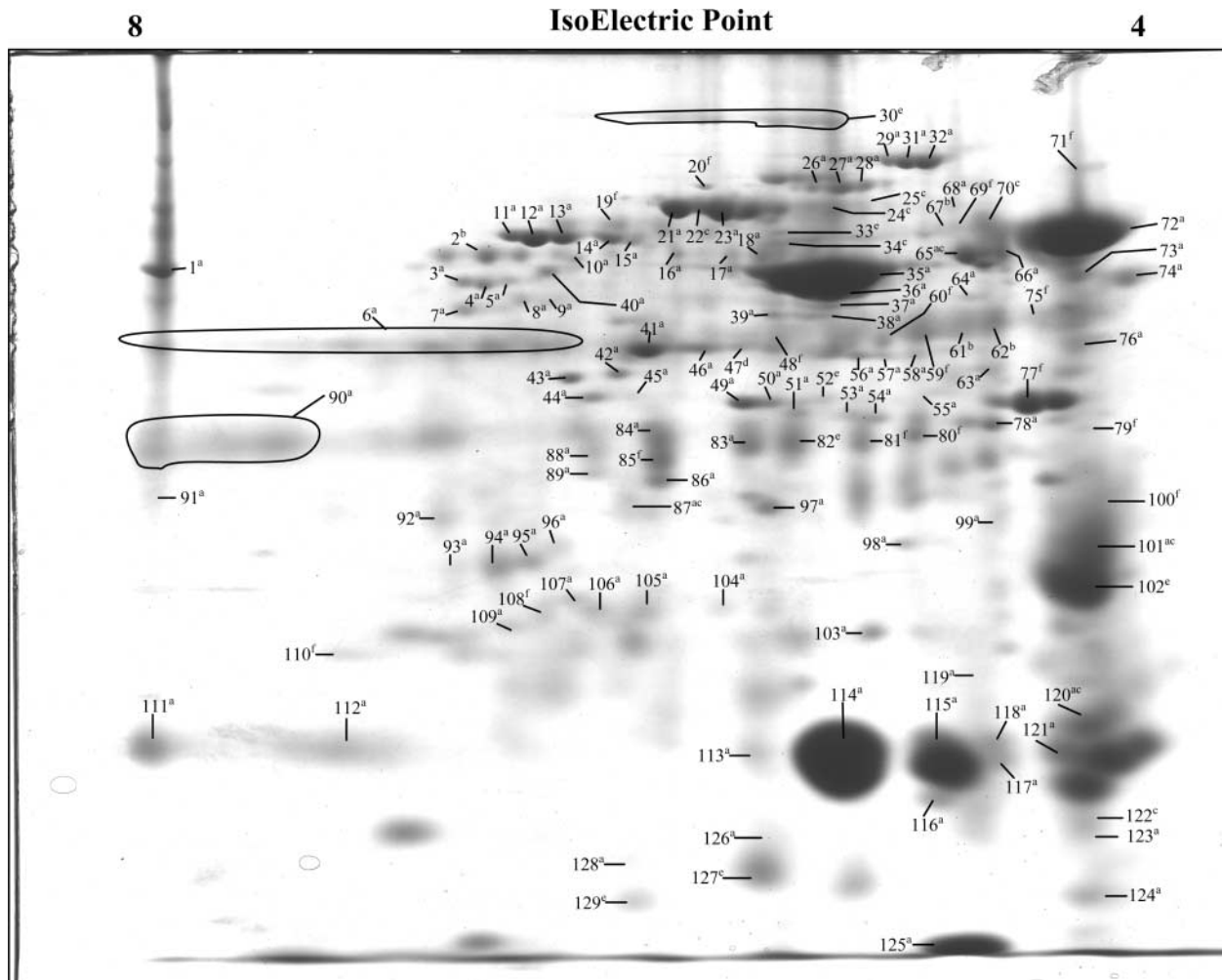


Figure 2 A representative protein spot map showing those intrauterine proteins that were identified by MS-Homology using *de novo* sequence information from MS/MS data. Illustrated are ^aproteins that increased or ^bdecreased due to day ($P < 0.05$ or 0.01), ^cincreased or ^ddecreased due to pregnancy status, or differed due to a day by status interaction (^eproteins increased at day 13 of pregnancy, and ^fall other interactions).

protein expression differences below this concentration was limited. Thus, we do not suggest that the spots identified fully represent the complete number or classes of secreted proteins by the uterus and/or conceptus during this period. However, this level of detection was suitable to identify the major proteins in uterine fluid that account for the overall increase in total uterine proteins between day 10 and 13 of the estrous cycle or pregnancy as previously shown (Vallet *et al.* 1998a).

In conventional 2D-PAGE studies, low-abundant proteins are difficult to detect as distinct spots because overwhelming quantities of more abundant and soluble proteins either prevent low-abundant proteins from being solubilized or obscure them on 2D-PAGE (Herbert *et al.* 2003). Removal of the more abundant proteins, e.g. albumin, from a sample prior to 2D separation may allow for greater visualization of low-abundant proteins. However, we elected to not remove albumin from our samples prior to 2D-PAGE, primarily because changes in the expression pattern of albumin isoforms, which affect ligand binding, may be indicative of

an individual's genotype (Ibeagha-Awemu *et al.* 2004), health status (Parkhomenko *et al.* 2002) or physiological state as shown in this study. Indeed, numerous spots that increased between day 10 and day 13 post-ovulation were identified as isoforms of serum proteins, confirming that in early gestation, proteins also found in serum constitute a significant portion of the total uterine luminal fluid proteins (Lee *et al.* 1998). These results indicate the extent that the intrauterine environment can be modified by proteins produced at remote sites (e.g., sites other than endometrium). It has been reported that secretion of conceptus estrogen at chorionic adhesion sites resulted in distinct morphological changes in the uterine epithelium underlying the conceptus and increased vascular permeability (Keys *et al.* 1986, Keys & King 1988, 1990), possibly explaining the increase in luminal serum proteins reported here. However, our results suggest that another mechanism is also responsible for the transudation of serum proteins into the uterus between day 10 and 13, based on the increase in serum proteins in cyclic gilts.

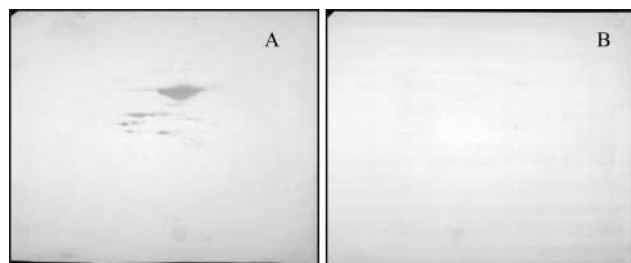


Figure 3 A representative immunoblot using rabbit anti-human serum albumin (A) or normal rabbit serum (B, control) to detect albumin present in the uterus at day 13 of pregnancy in gilts is illustrated. Multiple isoforms of albumin detected using a specific albumin antibody confirmed most of the results of the database search using MS/MS-derived data.

The uterus, during periods of increased progesterone, synthesizes binding proteins that facilitate the transport of nutrients to the developing conceptus (Vallet *et al.* 1996). For example, RBP, a member of the lipocalin family of proteins that is secreted by the uterus, participates in the delivery of retinol to the fetus (Vallet *et al.* 1996, 1998a, Vallet 2000). In this study, we found 11 spots that were identified as RBP. Of these, six spots are consistent with the four charged states of 22 kDa RBP previously published (Stallings-Mann *et al.* 1993), a result that helps to confirm the validity of our use of mass spectrometry to identify proteins. One higher molecular mass spot (number 77) was identified as RBP and matrix metalloproteinase (MMP) on different gels. Stallings-Mann *et al.* (1993), using Western blots, confirmed the presence of a minor 32–33 kDa band following anion exchange chromatography of separated RBP isoforms, thus supporting the possibility that spot 77 is an isoform of RBP. Interestingly, a second group of proteins were identified as boar salivary lipocalin (spots 121 and 122). The boar salivary lipocalin sequence is published and the sequence shares short conserved sequence motifs with all lipocalins (Flower 1996). Based on spot location after 2D-PAGE, the protein we identified as boar salivary lipocalin is similar to a 19 kDa lipid-binding protein secreted by the mare uterus in response to increasing progesterone and pregnancy (Crossett *et al.* 1998, Stewart *et al.* 2000). In the equine, uterine lipocalin binds many small lipids, including oleic, arachidonic, linoleic, linolenic, docosahexaenoic and eicosapentaenoic acids (Stewart *et al.* 2000) that are essential to the cellular differentiation and development of the mammalian embryo (Dutta-Roy 2000). Given the significant increase of boar salivary lipocalin in the uterus and its possible role in lipid transport, it is intriguing to speculate on the role of lipid metabolism during early conceptus development in the pig.

We also identified two proteins in the uterine luminal fluid that are involved in sphingolipid metabolism. We identified five isoforms of prosaposin, a family of lysosomal sphingolipid activator proteins that facilitate the catabolism of glycosphingolipids to ceramide (Munford *et al.* 1995).

The expression of four prosaposin isoforms was significantly enhanced in the day 13 pregnant samples, suggesting that the conceptus may be the primary source for these proteins. In addition, we identified two isoforms of *N*-acylsphingosine amidohydrolase (acid ceramidase) that increased during both the estrous cycle and early pregnancy. Acid ceramidase catalyzes the degradation of ceramide to sphingosine and liberates the fatty acid side chain. The abundance of these proteins in uterine flushings suggests that secretion of prosaposins and acid ceramidase by the conceptus and uterus respectively may occur in order to modify glycolipids to facilitate fetal development and/or trophoblast elongation, implantation or embryo development. Ceramide-based lipids are inserted in the plasmalemma with the oligosaccharide side chains extending into the extracellular environment, positioning these compounds to interact with both ligands and receptors (Yates & Rampersaud 1998, Tettamanti 2004). Modification of the hydrophilic domain of glycolipids affects the ability of integrins to bind with their receptors, thus potentially affecting implantation. Interestingly, we also identified two glycosyl hydrolases, *N*-acetyl- β -glucosaminidase and α -*N*-acetylgalactosidase, that increased between day 10 and day 13 of the estrous cycle and in early pregnancy. Glycosyl hydrolases sequentially remove individual sugar residues starting at the non-reducing terminal and result in the final end product, ceramide (Tettamanti 2004). The exact role of these proteins in the uterus during this period is unknown; however, modification of glycolipids could increase or inhibit the binding of cytokines or growth factors to the membrane (Yates & Rampersaud 1998) or play a role in trophoblast elongation or attachment (Burghardt *et al.* 2002).

In this study, spot 91, which increased in the uterine lumen by day 13 post-ovulation, was identified as uteroferrin. The increase in uteroferrin during the cycle and early pregnancy and the position of this protein on 2D-PAGE is consistent with previous studies (Geisert *et al.* 1982a, Roberts & Bazer 1988, Vallet *et al.* 1998a), providing further validation of the approach used to identify proteins. Uteroferrin functions to transport iron to the fetus (Roberts & Bazer 1988), which is considered essential for normal fetal development. However, iron-containing proteins catalyze lipid peroxidation in the presence of H_2O_2 and ascorbic acid (Minotti 1993, Vallet 1995). Lipid peroxidation has been associated with cell death and has been shown to restrict intrauterine growth of mammalian fetuses (Karowicz-Bilinska 2004). The toxic effects of iron on the fetus, due to lipid peroxidation, may be regulated by a concomitant increase in transferrin, which binds excess iron (Vallet *et al.* 1996), and by RBP, which may provide antioxidant activity in the form of retinol. In this study, we identified seven isoforms of transferrin. The position of some of these isoforms is consistent with previous immunoblotting results (Vallet *et al.* 1996). Three isoforms (spots 11–13, Fig. 2) had similar molecular masses but different charged states. The four lower

Table 3 Interpretation of the biological relationships among the identified uterine proteins using PANDORA classification of SwissProt keywords according to Gene Ontology annotation.

Protein classification	Spot number(s)	GenBank accession number
Serum transport/binding proteins		
Lipid transport/binding		
Albumin	27*, 35–38, 39* [†] , 40–42, 43* [†] , 44–58, 59*, 63*, 78*, 80, 94*, 103, 106, 107*, 109*, 123*, 124*, 128*, 129*	113578
Retinol-binding protein	77*, 113–117, 118*, 119*, 123*, 124*, 401*	3041715
Salivary lipocalin	118*, 120, 121	20178087
Annexin IV	98	71768
Iron-binding proteins		
Transferrin	11–17, 18* [†]	136192
Uteroferrin	91	417521
Inhibitor for carbonic anhydrase	18*, 34	6016307
Melanoma-associated antigen p97	67*, 69*	5174559
Other-binding proteins		
Connective tissue growth factor	123*, 127*	3023580
Folate-binding protein	92	4928857
Lipid metabolism		
N-Acylsphingosine amidohydrolase	90, 125	30089928
Prosaposin	100–102, 119*, 127*	13878928
Palmitoyl-protein thioesterase	87	27806465
Oxidoreductases		
Superoxide dismutase	93, 94*, 95, 104, 105	1711431
Peptidyl-glycine alpha-amidating monooxygenase	96	23503036
Glutathione-S-transferase	110*	544445
Quiescin Q6	1*, 8*, 9*	13325075
Carbohydrate metabolism		
Alpha-amylase	64	67374
Alpha-N-acetylgalactosamidase	63*	3396057
N-Acetyl-beta-glucosaminidase	4*, 5*	6225504
Fucosidase	60	14042931
Triosephosphate isomerase	107*, 108*, 109*	136062
Villin-2	33	27806351
N-Acetylglucosamine-6-sulfatase	19, 20*	1707906
Malate dehydrogenase	97	6226874
Protein metabolism and modification		
Proteases		
Metalloproteases		
Meprin	70*, 71*, 72, 74*	5174551
Matrix metalloproteinase	7* [†] , 65, 66* [†] , 69*, 76* [†] , 77, 78	15419710
Cysteine protease		
Cathepsin C	7*	30038325
Cathepsin L	82–86, 88, 89	2499874
Complement factor D	107*, 108*	3915626
Serine protease		
Haptoglobulin	79*	41019122
Protease inhibitors		
Serine protease inhibitors		
Alpha-2-macroglobulin	9* [†] , 10* [†] , 18*, 21–30, 31* [†] , 68, 118*	6978425
Alpha-1-inhibitor III	88, 31*, 32	112893
Activated T cell marker	73*, 74*	37359236
Complement C3	3, 4*, 5*	11869931
Inter-alpha-trypsin inhibitor heavy chain	62*	3024051
Alpha-1-antichymotrypsin	62*	9955853
Cysteine protease inhibitor		
Alpha-2-HS glycoprotein	Not shown	231467
Metalloprotease inhibitor		
Tissue inhibitor matrix metalloprotease-2	111* [†] , 112* [†]	3982745
Immune function		
Ig-heavy chain	6, 39*, 43*, 59*, 61	5052050
CH ₄ -secreted domains of IgM	40*, 68*, 69*, 70*	1522767
Poly-Ig-receptor	2, 10*	6863080
Structural/cellular proteins		
Programmed cell death		
Clusterin	99	1619636
HP 95	20*	303831
Intracellular protein trafficking		
Syntaxin-binding protein	1*	67415
Chordin-like protein	128*	34147715
Syndecan-binding protein	94*	2795863
Structural transmembrane proteins		
Actin	78*	71611
Interferon-induced transmembrane	129*	32480628

*Indicates spots with more than one protein identified based on MS-Homology search from different gels.

†Indicates protein identity confirmed using immunoblot.

molecular mass isoforms are more neutral proteins suggesting cleavage of side chain(s) from these isoforms. The physiological importance of these putative side chains remains unclear. In addition to transferrin and RBP, four additional proteins with antioxidant/protective properties that increased between day 10 and day 13 post-ovulation were detected in our study. Of these, superoxide dismutase (SOD) was the most abundant, based on number of spots and summed spot intensity. Previous reports suggest that increased SOD and decreased radical generation in the uterus are associated with increased progesterone levels, whereas estrogen induces superoxide radical production associated with decreasing SOD levels at the time of implantation in mice (Laloraya *et al.* 1996, Jain *et al.* 1999). Our results are consistent with the concept that antioxidant levels within the uterus increase under the influence of progesterone, thus protecting the uterine environment from the deleterious effects of free radicals associated with increased estrogen production by the conceptus, conceptus metabolism of lipids during maternal recognition of pregnancy or the possible deleterious effects of uteroferrin.

Proteins involved with protein metabolism and protease inhibitors made up the largest group of proteins found in this study. These proteins play an important role in determining the extent of trophoblast invasion through mechanisms that modify the integrity of the uterine or conceptus glycocalyx during embryo migration and superficial attachment (Salamonsen 1999, Esadeg *et al.* 2003, Aplin & Kimber 2004). We identified two uterine matrix MMP proteins, mepirin and MMP-2, that increase between day 10 and 13 of the estrous cycle and pregnancy, and seven isoforms of cathepsin L, a cysteine protease. Our results show that the majority of protease isoforms (13/20) were secreted irrespectively of conceptus presence. However, the remaining protease isoforms were greater in pregnant uteri, suggesting that the conceptus, either directly or indirectly through the stimulation of the uterus, increased secretion of these isoforms. We also identified a cysteine peptidase, complement factor D that is an important catalyst in the activation pathway of complement component C3, a protease inhibitor. Our results indicate that the most abundant group of protease inhibitors present during the estrous cycle and early pregnancy belong to the alpha-2 macroglobulin family: α -1-inhibitor-III (α 1I), C3 and α -2-macroglobulin (α 2M). Our results show that 15 spots corresponding to α 2M significantly increased between day 10 and day 13, regardless of conceptus presence. These results suggest that α 2M presence in the porcine uterus is regulated by progesterone. The existence of several isoforms of each protease inhibitor suggests that α 2M, C3 and α 1I are modified following translation. One cluster of α 2M spots (spot 30) increased only in the pregnant gilt. The source of these isoforms is unknown. We also identified two spots as tissue inhibitor of MMP-2 (TIMP-2), an endogenous inhibitor of MMP-2. Menino *et al.* (1997) demonstrated that TIMP-2 is

expressed by the swine embryo and uterus at day 15. Synthesis of TIMP by these two tissues could explain the two isoforms we detected where spot 111 increases at day 13 in the uterus of pregnant gilts and spot 112 increased in the uterus between day 10 and 13 in both the cyclic and pregnant uteri. Secretion and activation of protease inhibitors probably maintain a balance with proteases and may provide intrinsic mechanisms for the maintenance of the extracellular matrix that regulate embryo attachment and spacing within the uterus.

In summary, we characterized the global changes occurring in the major proteins within the intrauterine environment during the estrous cycle and pregnancy between day 10 and 13 postovulation using mass spectrometry following 2D-PAGE. The validity of the protein identifications obtained is indicated by (i) our demonstrated ability to appropriately match spots between gels, (ii) our ability to confirm several of the protein identifications using Western blotting, and (iii) our correct identification of previously described or identified proteins, including uteroferrin, RBP, folate-binding protein, transferrin, serum albumin, and proteases and protease inhibitors. Our results confirm that the concentrations of most of the major proteins present within the intrauterine environment are independent of conceptus presence. Furthermore, we identified proteins that are involved in lipid metabolism that have not been previously reported during implantation in the pig. This is the first report demonstrating the presence of enzymes that are involved with sphingolipid metabolism. The role of soluble proteins involved with ceramide metabolism requires further investigation given that many of the components of this pathway, including ceramide and sphingosine-1-phosphate play roles in signal transduction and chemotaxis in other cell systems. In conclusion, mass spectrometry combined with 2D-PAGE provided a high-throughput means to identify proteins present in the intrauterine environment, providing insights into the physiological processes taking place between day 10 and 13 of the estrous cycle and pregnancy in swine.

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