Protein Expression of Mouse Uterus in Post-Implantation

Hong Rye Kim, Rong Xun Han, Myung Youn Kim, Yunfei Diao, Chang Sik Park and Dong Il Jin†

Division of Animal Science & Resources, Research Center for Transgenic Cloned Pigs, Chungnam National University, Daejeon 305-764, Korea

ABSTRACT

Pregnancy is a unique event in which a fetus develops in the uterus despite being genetically and immunologically different from the mother, and the underlying mechanisms remain poorly understood. To analyze the differential gene expression profiles in nonpregnant and 7 days post coitus (dpc) pregnant uterus of mice, we performed a global proteomic study by 2-D gel electrophoresis (2-DE) and MALDI-TOF-MS. The uterine proteins were separated using 2-DE. Approximately 1,000 spots were detected on staining with Coomassie brilliant blue. An image analysis using Melanie III (Swiss Institute for Bioinformatics) was performed to detect variations in protein spots between pregnant and nonpregnant uterus. Twenty-one spots were identified as differentially expressed proteins, of which 10 were up-regulated proteins such as alpha-fetoprotein, chloride intracellular channel 1, transgelin, heat-shock protein beta-1, and carbonic anhydrase II, while 11 were down-regulated proteins such as X-box binding protein, glutathione S-transferase omega 1, olfactory receptor Olfr204, and metalloproteinase-disintegrin domain containing protein TECADAM. Most of the identified proteins appeared to be related with catabolism, cell growth, metabolism, regulation, cell protection, protein repair, or protection. Our results uncovered key proteins of mouse uterus involved in pregnancy.

(Key words : Mouse uterus, Pregnancy, 2-D gel electrophoresis, Mass spectrometry)

INTRODUCTION

Pregnancy is a special event in which a fetus develops in the uterus despite being immunologically and genetically different from the mother (a hemi-allograft). How the allogeneic fetus escapes immune rejection during pregnancy is a major paradox in transplantation- and xenotransplantation-related research (Mellor and Munn, 2000; Thellin *et al.*, 2000). This matter has been receiving increasing attention in recent years and is now recognized to have major clinical consequences besides basic relevance for mammalian reproduction. The uterus, in particular, is a dynamic organ and undergoes distinct functional changes during the estrus cycle, implantation, pregnancy, and parturition. It has heterogeneous cell types which have unique functions in pregnancy. Adding to this complication is the fact that their actions change depending on their proximity to the implanting embryo and with progression of pregnancy (Burnum *et al.*, 2008). Thus, it is important to study uterine molecular changes using conventional approaches. Reports on mice have established that uterine receptivity occurs for a limited period on 4 days post coitus (dpc) (Paria *et al.*, 1993). Once implantation occurs, the uterine stromal cells surrounding the embryo undergo extensive remodeling, a process termed as decidualization. One function of the deciduum is to provide nutrition for the developing embryo before the organization of a functional placenta (Wang and Dey, 2006). It is predicted that protein signatures between implantation and interimplantation sites are very complicated. Even though researches on gene expression using *in situ* hybridization and immunohistochemistry have provided profiles of specific genes that are expressed during implantation and decidualization (Wang and Dey, 2006; Dey *et al.*, 2004), a large number of gene products that are produced in the uteri for maintaining pregnancy post-implantation remains poorly understood.

Currently, two-dimensional gel electrophoresis (2-DE) in combination with MALDI-TOF mass spectrometry is a frequently used technique to analyze the complex cellular proteome (Görg *et al.*, 2000; Kim *et al.*, 2005). It provides high-throughput information about the state of the gene products, post-translational modification, expression levels, and changes in the normal cells with parameters resulting from the effect of external factors or various physiological disorders. Until now, these proteomic approaches have not been used to analyze pregnant and nonpregnant mouse uteri.

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† Corresponding author : Phone: +82-42-821-5876, E-mail: dijin@cnu.ac.kr

In this study, we investigated the differential gene expression patterns between pregnant and nonpregnant mouse uteri. The experiment was performed using 2-DE and mass spectrometry to identify the uterine proteins associated with pregnancy.

MATERIALS AND METHODS

Mouse Uterus Samples

Adult ICR mice were purchased from Orient Bio Inc. (Seoul, Korea). To isolate the pregnant uteri, 6- to 8-week- old ICR female mice were housed with adult male mice and examined daily for vaginal plugs. The noon of the day on which a vaginal plug was found was designated as 0.5 dpc. Cesarean sections were performed at 7 dpc for pregnant and nonpregnant uteri, and the sections were immediately frozen and stored at −80℃ until use (Fig. 1).

Extraction of Solubilized Proteins from the Uterus

For 2-D PAGE, soluble proteins were extracted as previously described (Kim *et al.*, 2005). In brief, lysis buffer A [1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulphonyl fluoride (PMSF), protease inhibitor cocktail (Roche), 100 mM Tris-HCl, pH 7.0] for pH in the 3～10 range was applied to the uterine tissue at a volume equal to tissue weight (e.g., 100 μl buffer/100 mg wet weight), and the samples were suspended with sonication for 15 sec and then chilled in ice. Lysis buffer B (7 M urea, 2 M thiourea, 4% CHAPS, 0.1 M DTT, 1 mM PMSF, protease inhibitor, 40 mM Tris-HCl, pH 7.0) was then added to the suspended samples. The samples were shaken gently for 1 hr at room temperature. The solubilized protein extracts were quantified by using the Bradford protein assay (Bio-Rad, USA), and the final protein samples were stored at −70℃.

2-D Gel Electrophoresis

Precast 18-cm IPG strips with pH in the 3～10 range were obtained from Amersham Biosciences. One milligram of the prepared protein samples was used for isoelectric focusing (IEF). The samples was mixed with rehydration buffer (6 M urea, 2 M thiourea, 4% CHAPS, 0.4% DTT, 2% v/v IPG buffer pH 3～10) to obtain a total volume of 450 μl (Görg *et al.*, 2000). The sample mixture was loaded onto IPG strips (pH 3～10; 180×3×0.5 mm). The strip was allowed to rehydrate overnight in a swelling tray. After rehydration, first dimensional IEF was performed using an Amersham Pharmacia Multiphor II IEF unit. Automatic isoelectric focusing was carried out at 1.5×10^5 Vh. The voltage was started at 100 V and then gradually increased to a final voltage of 8,000 V. Next, the IPG gel strips were placed in an equilibration solution (6 M urea, 2% SDS, 50% v/v glycerol, 2.5% acrylamide, 1.5 M Tris-HCl, pH 8.8) containing 5 mM TBP for 20 min with gentle shaking. Second dimensional separation was performed on 8～16% linear gradient SDS polyacrylamide gels. The gels were placed in an ISO-DALT system (Hoefer Scientific Instruments, San Francisco, CA, USA). The gels (200×250×1.0 mm) were run overnight at 10～15 mA until the bromophenol blue marker dye disappeared from the bottom of the gel.

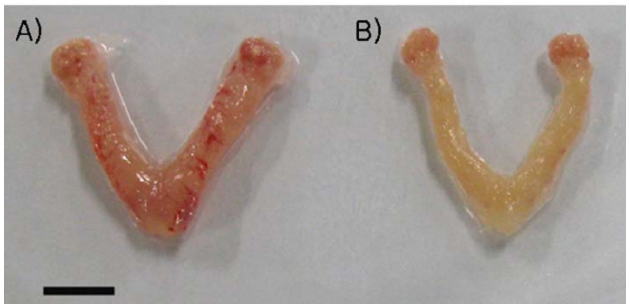

Fig. 1. (A) Representative photographs of the pregnant uterus collected on 7 dpc. (B) Photographs of the nonpregnant uterus. Black bar=5 mm.

Staining and Image Analysis of 2-DE

After 2-DE, gels for mass spectrometry were stained using colloidal Coomassie brilliant blue (CBB) G-250. The gels were fixed for 1 hr in a fixation solution (30% v/v methanol, 10% v/v acetic acid) and stained with colloidal CBB G-250 for 24 hr followed by destaining with 1% acetic acid. These gels were then analyzed using Melanie III software (Swiss Institute for Bioinformatics, Geneva, Switzerland). These calculations were applied to the percent volume (% volume of spot: OD×area×100) parameter, representative of protein expression. Variations in abundance were calculated as the ratio of average values (% vol) for a group between pregnant and non-pregnant uterus. The process for the validation of variant proteins was carried out by human operators (IImam-Sghiouar *et al.*, 2002).

Sample Preparation for MALDI-TOF Mass Spectrometry Analysis

In-gel digestion of proteins was performed as previously described (Shevchenko *et al.*, 1996) with minor modifications. For the CBB-stained proteins, the gel slab was destained using 120 μl wash solution (50% v/v acetonitrile, 25 mM NH_4HCO_3, pH 7.8). The gel pieces were then dehydrated with 50 μl of acetonitrile and dried for 30 min in a vacuum centrifuge. The dried gel pieces were rehydrated with 5 μl trypsin solution (trypsin at a concentration of 0.0012 μg/μl in 25 mM NH_4HCO_3, pH 7.8). If needed, additional ammonium bicarbonate buffer was added to completely cover the gel pieces. The digestion was performed at 37℃ overnight. After

complete digestion, the supernatant was transferred to another Eppendorf tube. To extract the residual peptides, the gel pieces were sonicated for 20 min at 30℃ in a solution of 50% acetonitrile/0.5% trifluoroacetic acid (TFA). The extracted peptides were analyzed using MALDI-TOF. After extracting the peptides, mass spectrometric analysis of peptide mass fingerprinting (PMF) was performed using a Voyager-DE STR MALDI-TOF-MS (PerSeptive Biosystems, Framingham, MA, USA). Approximately 1 μl of the extracted peptide solution from each gel spot piece and the same volume of a matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid, 0.1% v/v TFA, and 50% v/v acetonitrile) were loaded onto a MALDI sample plate (96-well plate) and crystallized. For each sample, an average of 500 spectra was obtained, and the scans were performed twice. The spectra were calibrated upon acquisition automatically using an external 3-point calibration. Peak assignment was performed manually using DataExplorer™ software that is part of the Voyager-DE STR MALDI-TOF-MS software package (PerSeptive Biosystems, Framingham, MA, USA) and the spectra were saved as peak table files (*.pkt) to search against a non-redundant protein sequence database on the internet [NCBInr (2009/11/03) Data Bank].

RESULTS

Analysis of Uterine Protein Pattern by 2-DE

Discrepancy in the uterine protein expression between pregnant and nonpregnant mice (Fig. 1) was analyzed using 2-DE. To show the molecular-level differences in protein expression that might be related to maintaining pregnancy and maternal tolerance, we compared the protein expression patterns of 2 uteri each of pregnant (7 dpc) and nonpregnant mice (control). The total protein of the uteri was isolated under the same conditions. The 2-DE maps obtained after the separation of each 1 mg of the protein sample from pregnant and nonpregnant uteri are shown in Fig. 2. Using the MALANIE III software (Swiss Institute for Bioinformatics, Geneva, Switzerland), we detected approximately 900～1,000 protein spots per gel. Image analysis of the 2 series of CBB-stained gels permitted us to analyze the spots and their level of expression in detail (% volume). The spots in Fig. 1A represent up-regulation, while those in Fig. 1B represent down-regulation (U: pregnant uterus up-regulation, D: pregnant uterus down-regulation). The enlargement of differential protein spots in the pregnant and nonpregnant uteri are shown in Fig. 3. On comparing the series of gels obtained from pregnant and nonpregnant uteri samples, we detected at least 23 protein spots that differed in intensity; of these 11 spots were up-regulated proteins in the pregnant uterus while 12 were down-regulated proteins.

Identification of Differentially Expressed Spots

To identify the differential expression spots in the two samples, we excised the uterine proteome gels to obtain

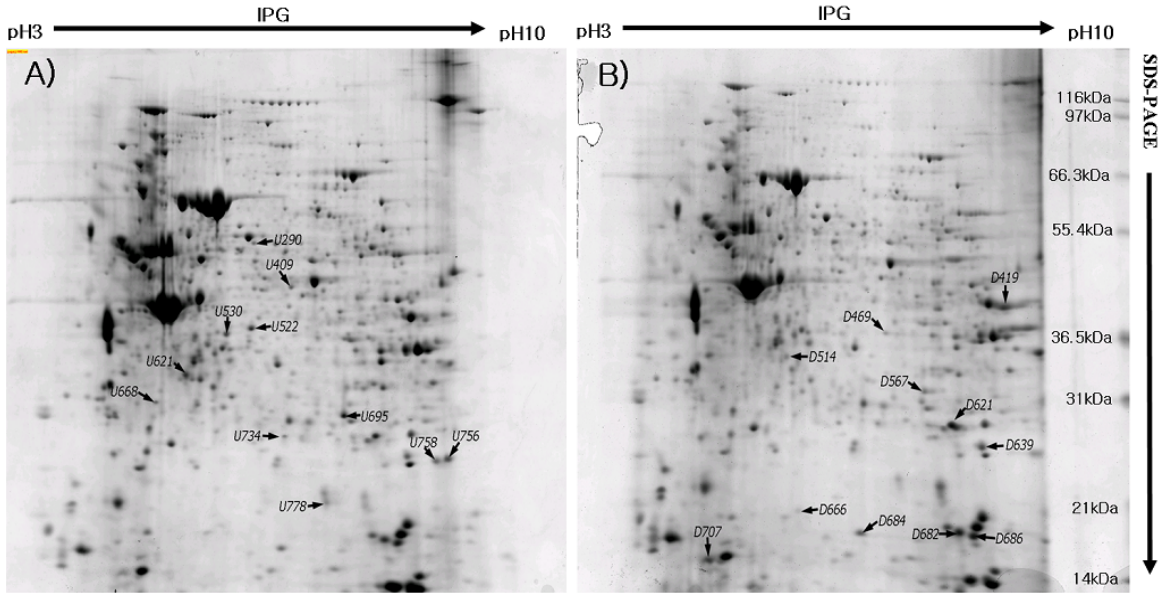

Fig. 2. 2-DE protein separation of pregnant and nonpregnant uterus as visualized by Coomassie brilliant blue staining. The first dimension was an 18-cm pH 3～10 non-linear IPG strip, and the second dimension was an 8～16% gradient gel. This is the separation of 1 mg of the extract. A mean of 900 spots were enumerated with MELANIE 3 software. Comparison of the proteomes of pregnant and nonpregnant uteri. The spot represents expression variations in the pregnant ((A), (7 dpc)) and nonpregnant (B) uteri.

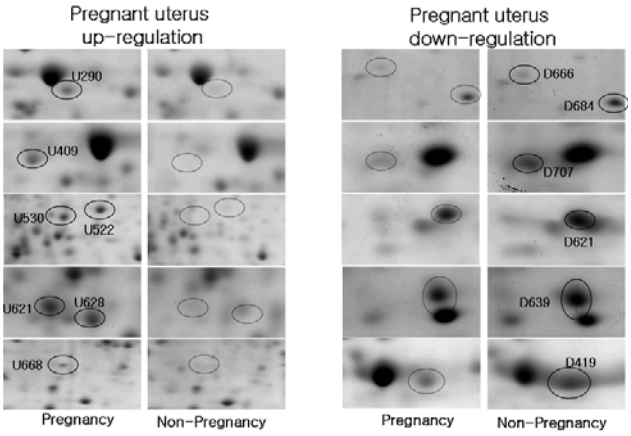

Fig. 3. Close-up sections of 2-DE protein patterns of pregnant and nonpregnant uteri. The black dotted line represents down-regulation, and the solid line represents up-regulation in the pregnant uterus.

the spectra of each peptide after tryptic digestion by MALDI-TOF-MS. The unprocessed spectra were processed by DataExplorer™ and analyzed using the lists of monoisotopic peaks. The tryptic peptide masses were used to look for protein candidates using the web-based searching software ProFound (http://129.85.19.192/profound_bin/WebProFound.exe). Twenty-one spots were identified as known proteins recorded in NCBInr (2009/11/03) databases. The search results were evaluated on the basis of accepted standards that take into account the number of peptides matched to the candidate protein, difference in the number of matched peptides between the candidate protein and the next best fit, coverage of the candidate protein's sequence by the matching peptides, and agreement of the experimental and theoretical pI and M_r with the obtained values [Jensen *et al.*, 1999]. The data pertaining to the identified proteins are summarized in Tables 1 and 2. The spots correspond to the identified proteins in Table 1 and Table 2 are indicated in Fig. 2.

Characteristics of Identified Proteins

On comparing the pregnant and nonpregnant uterus samples, we found that a total of 21 protein spots were expressed differentially, of which 10 were up-regulated proteins such as such as alpha-fetoprotein, chloride intracellular channel 1, transgelin, heat-shock protein beta-1, and carbonic anhydrase II, and 11 spots were down-regulated proteins such as X-box binding protein, glutathione S-transferase omega-1, olfactory receptor Olfr-204, peptidylprolyl isomerase A, superoxide dismutase 1, triosephosphate isomerase, and metalloproteinase-disintegrin domain containing protein TECADAM. Two spots could not be identified. According to the annotation from UniProt Knowledgebase (Swiss-Prot/TrEMBL) and Gene Ontology Database, the proteins could be divided into several groups (Table 1 and 2). Proteins involved in regulation of transcription, heme biosynthesis, transport, glutathione transferase activity, muscle organ development, embryo implantation, chloride transport, one-carbon metabolic process, stress response, blood coagulation, integrin-mediated signaling pathway, and G-protein coupled receptor protein signaling pathway are differentially expressed between 7 dpc pregnant and nonpregnant uteri.

Table 1. Identification of up-regulated proteins in the pregnant uterus

Spot ID	Est'd Z[a)]	Accession No.	Protein information	%	pI	kDa	Protein category
U290	2.22	NP_862897.1	Fibrinogen beta chain precursor	34	6.7	55.59	Blood coagulation
U409	1.59	AAA37190.1	Alpha-fetoprotein	19	5.5	49.2	Transport
U522	2.06	AAA37190.1	Alpha-fetoprotein	16	5.5	49.2	Transport
U530	2.33	AAA37190.1	Alpha-fetoprotein	18	5.5	49.2	Transport
U621	1.55	P07724.3	Serum albumin; Flags: Precursor	18	5.8	71.23	Transport
U688	2.38	NP_254279.1	Chloride intracellular channel 1	36	5.1	27.42	Chloride transport
U695	2.31	AAA37356.1	Carbonic anhydrase II	52	6.5	29.22	One-carbon metabolic process
U756	2.4	NP_035656.1	Transgelin	58	8.8	22.63	Muscle organ development
U778	1.22	NP_035656.1	Transgelin	31	8.8	22.63	Muscle organ development
U734	1.93	P14602.3	Heat shock protein beta-1	41	6.1	23.07	Stress response

[a)] Z score is the distance to the population mean in units of standard deviation. It also corresponds to the percentile of the search in the random match population. Conceptually, this "95th percentile" is different from "95% confidence" and denotes that the search is a correct identification. (The following is a list for the Z score and its corresponding percentile in an estimated random match population: [(Z: percentile) 1.282:90, 1.645:95.0, 2.326: 99.0, 3.090: 99.9]).

Table 2. Identification of down-regulated proteins in the pregnant uterus

Sopt ID	Est'd Z	Accession No.	Protein information	%	pI	kDa	Protein category
D419	1.83	AAL60202.1	X-box binding protein processed isoform	34	4.7	40.15	Regulation of transcription, DNA-dependent
D469	1.93	NP_032551.3	Delta-aminolevulinic acid dehydratase	32	6.3	36.57	Heme biosynthetic process
D514	1.63	AAA37190.1	Alpha-fetoprotein	21	5.5	49.2	Transport
D567	2.05	NP_034492.1	Glutathione S-transferase omega 1	36	6.9	27.76	Glutathione transferase activity
D621	1.95	P17751.3	Triosephosphate isomerase	31	6.9	27.12	Embryonic development
D639	1.94	1GLP_A	Chain A, 1.8 angstroms molecular structure of mouse liver class Pi glutathione S-transferase complexed with S-(P-nitrobenzyl) glutathione and other inhibitors	44	8.3	23.67	Glutathione metabolic process
D666	1.33	NP_667203	Olfactory receptor Olfr204	20	9.6	35.16	G-protein coupled receptor protein signaling pathway
D682	1.18	NP_032933.1	Peptidylprolyl isomerase A	30	7.9	18.17	Protein folding
D684	2.14	NP_035564.1	Superoxide dismutase 1, soluble	39	6	16.14	Embryo implantation
D686	1.07	NP_032933.1	Peptidylprolyl isomerase A	26	7.9	18.17	Protein folding
D707	1.07	AF163293_1	Metalloproteinase-disintegrin domain containing protein TECADAM	8	6.6	75.41	Integrin-mediated signaling pathway

DISCUSSION

The molecular basis and interaction between the uterus and fetus for immunological tolerance remains poorly understood and the regulatory mechanisms are unclear. Recent reports have shed some light on this process, and various genes have been identified to be essential in preparing the uterus for maintaining pregnancy, uterine cell lineages, and fetal survival. Although researches on gene expression using *in situ* hybridization and immunohistochemistry have provided the profiles of specific genes during implantation and pregnancy, this mechanism in relation to maternal tolerance and maintaining pregnancy requires studies that are more on a molecular level. We conducted a proteomics study in which we compared 7-dpc pregnant and nonpregnant mice uteri because 7 dpc is a stage where there are rapid trophoblast invasion and implantation swellings (Kennedy *et al.*, 2007). The expression levels of 22 protein spots differed significantly between the 2 kinds of uteri. The threshold, 2 times up- or down-regulation, was chosen arbitrarily to exclude proteins that differ in integrated intensity due to small variations occurring randomly during the experimental setup. In our experiment, the expression of heat-shock protein beta-1 was higher in the pregnant uteri than in the nonpregnant uteri. The expression level of HSP 27 increased in pregnant uterus. This can be attributed to reduced activated oxygen production and to the resultant excitation of a stressed state of hypoxia in the placenta and uterus. Heat-shock protein 27 (HSP 27) was earlier demonstrated to be related with matrix metalloproteinase (MMP) activity. HSP 27 is a downstream effector of p38 mitogen-activated protein (MAP) kinase-mediated MMP-2 activation and cell invasion in human prostate cancer (Xu and Bergan, 2006). Moreover, another report showed that HSP 27 levels increase in differentiated trophoblast cells and that placental exposure to 6-mercaptopurine (known inhibitor of extravillous trophoblast cell invasion) is associated with reduction in trophoblast HSP 27, iNOS, NO, and MMP-2 levels (Matalon *et al.*, 2005). The level of HSP 27 is increased in pregnant uterus. Thus, increase in HSP 27 levels contribute to rapid trophoblast invasion and implantation swellings in the post-implantation uteri, to overcome the increase in the uterine wall tension due to the growing embryo and pregnancy.

As listed and summarized in Table 1 and Fig. 1, several proteins could be identified as differentially expressed in the pregnant uterus. Although further research is required to isolate the unique uterine proteins using narrow range strips (pH 6~9, pH 4~7, one unit) and to study the regulatory mechanisms for the change in protein expression levels to understand the complicated mechanism of pregnancy, the present proteomic study shows for the first time that differential protein expression

between pregnant and nonpregnant uterus is closely related to the regulation of specific genes involved in maintaining pregnancy and maternal tolerance.

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