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Proteomic Analysis of Proteins Differentially Expressed in Uterine Lymphocytes Obtained From Wild-Type and NOD Mice

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ABSTRACT

Non-obese diabetic (NOD) mice exhibit impaired fertility and decreased litter size when compared to wild type (WT) mice. However, it is unclear why allogeneic pregnant NOD mice are prone to spontaneous embryo loss. Herein, two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) were used to detect differentially expressed proteins in the uterine lymphocytes isolated from these mice and WT BALB/c controls. We found 24 differentially expressed proteins. The differential expression of 10 of these proteins was further confirmed by Western blot analysis. Out of the 24 identified proteins, 20 were expressed in uterine lymphocytes of WT mice at a level at least 2 times higher than in NOD mice, whereas 4 were down-regulated. Western blot analysis confirmed that 8 proteins were up-regulated and 2 proteins were down-regulated in WT mice compared with NOD mice, consistent with the results of 2-DE and MS. Additionally, most of the highly expressed proteins in WT uterine lymphocytes were expressed at a significantly lower level in the corresponding splenic group (17/20). These results suggest that up-regulated expression of these proteins may be specific to uterine lymphocytes. Reported functions of the highly expressed proteins affect key functions during pregnancy, including cell movement, cell cycle control, and metabolisms. Finally, we analyzed the constitutional ratio of $CD3^+$ and $CD49b^+$ cells in the isolated lymphocytes by flow cytometry. Our results suggest that the differentially expressed proteins may participate in the modulation of embryo implantation and early-stage development of embryos, and subsequently influence pregnancy outcome. J. Cell. Biochem. 108: 447–457, 2009.

KEY WORDS: ANIMAL MODEL; IMMUNODEFICIENCY; PREGNANCY; PROTEOMICS

Non-obese diabetic (NOD) mice are commonly used as a mouse model of insulin-dependent type 1 diabetes mellitus. A considerable percentage of NOD mice are insulin-deficient due to the loss of pancreatic β-cells because of an autoimmune assault [Gowri et al., 2007; Lin et al., 2009]. A previous study demonstrated that insulin-dependent diabetes occurs spontaneously in 9% and 80% of female NOD mice by 12 and 29 weeks of age, respectively. Moreover, NOD mice show impaired fertility with poor implantation

Abbreviations used: 2-DE, two-dimensional gel electrophoresis; C1q, complement component 1q; ECL, enhanced chemiluminescence; eIF5a, eukaryotic translation initiation factor 5a; FABP5, fatty acid binding protein 5; GP1, glutathione peroxidase 1; HP1β, heterochromatin protein 1β; IPG, immobilized pH gradient; MLC2, myosin light chain 2; MS, mass spectrometry; MW, molecule weight; NOD, non-obese diabetic; PA28α, proteasome (prosome, macropain) 28 subunit α; PMF, peptide mass fingerprinting; PRX II, peroxiredoxin II; Rho GDI, Rho GDP dissociation inhibitor; WT, wild type.

Cui Li and Wenjing Wang contributed equally to this work.

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and low embryo viability, with a 50% smaller litter size in NOD mice than in non-immunodeficient controls [Formby et al., 1987]. This implies that approximately 50% of NOD mice embryos are rejected by the maternal immune system during pregnancy. This level is significantly higher than the 4–5% rejection frequency observed in non-immunodeficient murine strains due to chromosome abnormalities [Clark et al., 1998].

In our current study, placental lymphocytes were collected from BALB/c × C57BL/6 and NOD × C57BL/6 allogeneic pregnancy models, and the protein expression profiles of lymphoid cell lysates were analyzed by two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). Data were compared with that from splenic lymphocytes and the constitutional ratio of uterine $CD3^+$ and $CD49b^+$ cells among the isolated lymphocytes was analyzed using flow cytometry. Twenty-four proteins were found differentially expressed. Out of these identified proteins, 20 were expressed in uterine lymphocytes of WT mice at a level at least 2 times higher than in NOD mice, whereas 4 were down-regulated. Additionally, most of the highly expressed proteins in WT uterine lymphocytes were expressed at a significantly lower level in the corresponding splenic group (17/20). These results suggest that up-regulated expression of these proteins may be specific to uterine lymphocytes. The possible role of these proteins may play during pregnancy are discussed.

MATERIALS AND METHODS

ALLOGENEIC PREGNANCY MODELS OF BALB/C × C57BL/6 AND NOD × C57BL/6

Female BALB/c and NOD mice and male C57BL/6 mice (8–12 weeks old) were purchased from the Experimental Animal Center of Zhongshan University (Guangzhou, China), and housed under specific pathogen-free conditions. The immunodeficiency of NOD mice was confirmed using methods described previously [Lin et al., 2005; Rocha-Campos et al., 2006]. BALB/c mice were used in the present study as wild-type (WT) controls. The abortion (resorption) sites were identified by their small size accompanied by a necrotic, hemorrhagic appearance, compared with normal embryos and placentas. The percentage of resorption was calculated as the ratio of resorption sites and total implantation sites (resorption plus normal implantation sites) as described previously [Lin et al., 2005, 2006]. All animal procedures followed the national animal care guidelines, and all related data was approved for publication by the University's Institutional Review Board.

PREPARATION OF UTERINE AND SPLENIC LYMPHOCYTE LYSATES

Uterine lymphocyte lysates from NOD mice and age-matched non-immunodeficient WT BALB/c mice were prepared as described previously [DiGiovanni et al., 2000]. Briefly, hysterolaparotomy was performed on gestational day 9.5 to collect embryo-depleted placentas together with decidua basalis. The pooled placentas and decidua basalis were carefully cut into small pieces and then collected in 0.9% NaCl solution and filtered through a 50-μm pore size nylon mesh to obtain a mononuclear cell suspension. Mononuclear cells were purified by centrifugation of cell suspensions on ficoll-hypaque density medium. Any contaminating red blood cells that might have persisted in the single-cell suspension were eliminated by two incubations with red blood cell lysis buffer at 37°C. The cells were then suspended in modified RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM ethylendiaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 0.66 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, 1 mM NaF), and sonicated three times in 5-s bursts. The cell lysates were centrifuged at 14,000 *g* for 15 min at 4°C. Protein concentrations in the supernatants were measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Single cell suspensions of splenocytes were prepared using methods described elsewhere [Lin et al., 2006]. Lymphocytes were purified by centrifugation of cell suspensions on ficoll-hypaque density medium. The cells lysates were then prepared as described above.

TWO-DIMENSIONAL GEL ELECTROPHORESIS (2-DE)

2-DE was performed according to the manufacturer's instructions. Samples were loaded onto linear immobilized pH gradient (IPG) strips (IPGstrip, pH 4-7 L, 180 mm × 3 mm × 0.5 mm, Amersham Biosciences). Briefly, 1 mg protein samples were diluted to 350 μl with a rehydration solution [7 mol/L urea, 2 mol/L thiourea, 2% CHAPS, 18 mM DTT, 0.5% (v/v) pH 4-7 IPG buffer, and trace bromophenol blue], and applied to IPG strips by 14 h rehydration at 30 V. The proteins were focused successively for 1 h at 500 V, 1 h at 1,000 V and 5 h at 8,000 V to give a total of 41,920 Vh on an IPGphor (Amersham Biosciences). Focused IPG strips were equilibrated for 15 min in solution (6 mol/L urea, 2% SDS, 30% glycerol, 50 mmol/L Tris–HCl, pH 8.8 and 1% DTT), and then for an additional 15 min in the same solution except that DTT was replaced by 2.5% iodoacetamide. After equilibration, SDS–PAGE was carried out at 10°C on 10% SDS slab gels using an Ettan DALT II system (Amersham Biosciences) with the IPG strips sealed on the top of the gels with 0.5% agarose. SDS–PAGE was run at constant power of 2 W/gel for 30 min, then switched to 12 W/gel until the bromophenol blue marker reached the bottom of the gel. After electrophoresis, the blue silver staining method (a modified Neuhoff's colloidal coomassie blue G-250 stain) was used to visualize the protein spots in the 2-DE gels [Li et al., 2006].

2-DE IMAGE DATA ANALYSIS

The stained 2-DE gels were scanned with LabScan software on Imagescanner (Amersham Biosciences) with 300 resolution. The spot-intensity calibration, spot detection, background abstraction, matching, 1-D calibration, and the establishment of average-gel were performed with PDQuest system (Bio-Rad Laboratories). Protein staining of individual spots was quantified by calculation of spot volume after normalization of the image using the total spot volume normalization method multiplied by the total area of all the spots. The calculation of the theoretical molecular weight (MW) and pI values of the identified protein spots is based on algorithms included in the PDQuest analysis software package. Proteins were classified as being differentially expressed when spot intensity showed a ≥2-fold variation in NOD in comparison to WT BALB/c. Significant differences in protein expression levels were determined using Student's *t*-test with a set value of $P < 0.05$ [Li et al., 2006].

PREPARATION OF PROTEIN SPOTS

Protein spots were excised from the preparative gels and placed into a 96-well microtiter plate. Proteins were in-gel digested as previously described [Yun et al., 2007]. The gel-spots were destained with destaining solution [200 mmol/L NH_4HCO_3 and 100% acetonitrile (ACN) (volume ratio 1:1)] for 20 min at room temperature. Gel spots were washed twice with deionized water, shrunk by dehydration in ACN and dried in a vacuum centrifuge. The samples were then swollen in a digestion buffer containing 20 mM ammonium bicarbonate and 12.5 ng/μl trypsin (Sigma, St. Louis) at 4°C. After a 30 min incubation, the gels were digested 12 h at 37°C. Tryptic peptides were extracted twice from the gel slices by 15-min sonication in 0.1% TFA/50% ACN solution. The supernatants were collected and dried to a pellet in a flow of high-purity nitrogen. The peptides were eluted with 0.7 μl CCA matrix solution (α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA and 50% ACN) and were loaded onto a stainless steel target with 192 wells (Applied Biosystems, Framingham, MA).

MASS SPECTRA ANALYSIS

Samples were allowed to air dry and were analyzed with the Applied Biosystems Voyager System 4700 MALDI-TOF–TOF Mass Spectrometer (ABI) using an Nd:YAG 200 Hz laser operating at 355 nm as the desorption ionization source. The spectra scan was performed in reflector-positive mode with an acceleration voltage of 20 kV. Positive ion mass spectra were recorded on a home built linear time-of-flight mass spectrometer using 39 keV of total acceleration energy. The initial MS scan utilized an m/z range of 700–3,500. During MS/MS analysis, air was used as the collision gas and collision energy was 1 keV. Spectra were obtained by accumulation of 500–3,000 consecutive laser shots. The TOF–TOF tandem mass spectra were acquired by the data-dependent acquisition method with the five most intense ions selected from one previous MS scan. External calibration was performed using a standard protein (myoglobin) digest before data acquisition.

PROTEIN IDENTIFICATION

Known contamination peaks such as keratin and autoproteolysis peaks were removed prior to database search. Spectra were processed and analyzed by GPS Explorer (Applied Biosystems) using the MASCOT software [Matrix Science, London, UK (http://www.matrixscience.com)] to search for peptide mass fingerprints and MS/MS data in the NCBInr database. The following parameters were used in the search: taxonomic category *all*; peptide mass tolerance, 0.2 Da; MS/MS ion mass tolerance, 0.6 Da; trypsin digest with one missing cleavage; variable modifications including methionine oxidation and cysteine carboxyamidomethylation. Protein scores by Mascot searching greater than 63 are significant ($P < 0.05$) [Li et al., 2006; Shen et al., 2007].

WESTERN BLOT ANALYSIS

Primary antibodies used in Western blot analysis include: goat anti-mouse eukaryotic translation initiation factor 5a (eIF5a) polyclonal antibody (1:100, Santa Cruz Biotechnology), goat anti-mouse fatty acid binding protein 5 (FABP5) (1:100, R&D Systems), rabbit anti-mouse glutathione peroxidase 1 (GP1) antibody (1:100, Abcam), rabbit anti-mouse heterochromatin protein 1β (HP1β) polyclonal antibody (1:100, Santa Cruz Biotechnology), goat anti-mouse proteasome (prosome, macropain) 28 subunit α (PA28α) polyclonal antibody (1:100, Santa Cruz Biotechnology), rabbit anti-mouse peroxiredoxin II (PRX II) polyclonal antibody (1:100, Santa Cruz Biothechnology), rabbit anti-mouse Rho GDP dissociation inhibitor (Rho GDI) (1:100, Cell Signaling Technology, Inc.), rabbit anti-mouse stathmin (1:200, Cell Signaling Technology, Inc.), rat anti-mouse complement component 1q (C1q) monoclonal antibody (1:100, Abcam), and rabbit anti-mouse myosin light chain 2 (MLC2) antibody (1:100, Cell Signaling Technology, Inc.).

Uterine lymphocytes from NOD mice and age-matched non-immunodeficient BALB/c mice were dissolved in lysis buffer (150 mM NaCl, 50 mM Tris–Cl pH 8.0, 0.1% NP-40, 1 mM PMSF, 25 μg/ml aprotinin, 25 μg/ml leupeptin), votexed, and incubated at room temperature for 2 h. The mixture was centrifuged at 15000 rpm for 30 min at 4°C. The concentration of the total proteins in the supernatants was assayed using the Bradford assay (Bio-Rad Laboratories). Western blotting analysis was performed as described [Li et al., 2006]. Briefly, 100 μg of total proteins were separated on 12% SDS–PAGE gels prior to transfer onto nitrocellulose membranes. After blocking with 5% milk in TBS/0.2% Tween 20 (TBS-T) for 1 h at room temperature, the blotting membranes were incubated with appropriate primary antibody listed above for 1 h at room temperature, followed by incubation in a 1:2,000 dilution of corresponding secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. NADPH was used as loading control. The reactions were visualized using enhanced chemiluminescence (ECL, Amersham Biosciences) detection system. Signals on the blots were visualized by autography.

DETECTION OF THE CONSTITUTIONAL RATIO OF $CD3^+$ AND $CD49B^+$ CELLS BY FLOW CYTOMETRY

Purified uterine lymphocytes from BALB/c and NOD mice at day 9.5 of gestation were incubated with FITC-conjugated anti-mouse CD45 and PE-conjugated anti-mouse CD3, or FITC-conjugated anti-mouse CD45 and PE-conjugated anti-mouse CD49b (all from eBioscience), washed once with PBS, and assayed on a FACS Calibur flow cytometer using CellQuest software (BD Biosciences, Franklin Lakes, NJ). Isotype controls were established by staining of isotype control antibodies (eBioscience) to exclude false positive cells [Lin et al., 2006, 2009]. All experiments were performed independently four times, and data are shown as mean ± 1 standard deviation (SD) [Li et al., 2006; Shen et al., 2007].

STATISTICAL ANALYSIS

Significant difference in protein expression levels were determined by Student's *t*-test with a set value of $P < 0.05$. Data are shown as mean ± SD. The number of repeated experiments in each design is indicated in the figure legend [Li et al., 2006; Shen et al., 2007].

RESULTS

THE PERCENTAGE OF EMBRYO LOSS

The percentage of resorbing embryos on day 9.5 of gestation in NOD × C57BL/6 mice (24.6%; n = 12; 35/142) was significantly

higher than that observed in WT BALB/c × C57BL/6 mice (3.2%; n = 16; 6/187; $P < 0.01$). This result suggests that allogeneic pregnant NOD mice are prone to spontaneous embryo loss.

ANALYSIS OF 2-DE PROTEIN MAPS IN NOD AND BALB/C MICE

Proteins from two sets of pooled splenic and uterine lymphocyte lysates from gestational day 9.5 in NOD mice and age-matched WT mice were resolved by 2-DE. Each set of pooled lysate contained samples from 12 separate mice. In order to validate reproducibility, 2-DE for the pooled NOD and WT splenic and uterine lymphocyte lysates was repeated three times under identical experimental condition and parameters. Following staining with coomassie brilliant blue, we obtained well-resolved and reproducible 2-DE maps from both the NOD and WT groups (Fig. 1). Analysis of quantitative spots with image analysis software from PDQuest detected 687 ± 43 protein spots in splenocytes and 621 ± 34 spots in uterine lymphocytes from NOD mice and 691 ± 51 spots in splenocytes and 589 ± 27 spots in uterine lymphocytes from WT mice.

A total of 24 protein spots showed a >2-fold decrease (20 spots) or increase (4 spots) in the NOD uterine lymphocyte group as compared to the WT uterine lymphocyte group. Moreover, 17/20 spots that were expressed at a significantly higher level in the WT uterine lymphocyte group were also significantly up-regulated when compared to the WT splenic lymphocyte group, suggesting that increased expression of these proteins may be specific to the pregnant uterus of WT mice. In contrast, spots 12, 14, and 18 were similarly expressed at a higher level in the WT splenic group as compared to the WT uterine group.

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS BY MALDI-TOF/TOF MS/MS

The 24 differentially expressed protein spots were excised from coomassie brilliant blue-stained gels and subjected to in-gel digestion with trypsin. An aliquot of the supernatant containing tryptic peptides was analyzed by MALDI-TOF/TOF MS/MS. High-quality peptide mass fingerprinting (PMF) and MS/MS spectrum were obtained. The MASCOT search program was used to identify the analyzed protein spots. Only the proteins ranked as the best hits with a significant score in the Mascot database were significant ($P < 0.05$) and identified as differentially expressed proteins. All 24 differentially expressed proteins were identified, including 20 down-regulated proteins and 4 proteins (myeloid bactenecin F1, capping protein muscle Z-line α2, myosin light polypeptide 6, and C1q) that were significantly up-regulated in the NOD uterine lymphocyte group compared with the WT uterine lymphocyte group (Table I). The representative mass spectra of spot 1, identified as eukaryotic translation initiation factor 5A (eIF5a), is shown in Figure 2.

Three spots (spots 12, 14, and 18) were identified that were expressed at a higher level in the WT splenic and uterine groups. Spot 12 represents Rho GDIβ, spot 14 represents Rho GDIα, and spot 18 represents capping protein muscle Z-line β.

The relative amounts of protein expression were obtained by comparing the density of protein spots in 2-DE map using a density detector (Table II). Among the 24 proteins analyzed, 20 were expressed at a significantly higher level (>2-fold; $P < 0.01$) in uterine lymphocyte lysates from WT mice compared to NOD mice, whereas the remaining 4 proteins were expressed at a significantly lower level in WT mice than in NOD mice. Furthermore, 6/24 proteins were expressed at a significantly higher level in splenic lymphocyte lysates from WT mice than in splenic lymphocyte lysates from NOD mice ($P < 0.01$) (Table II).

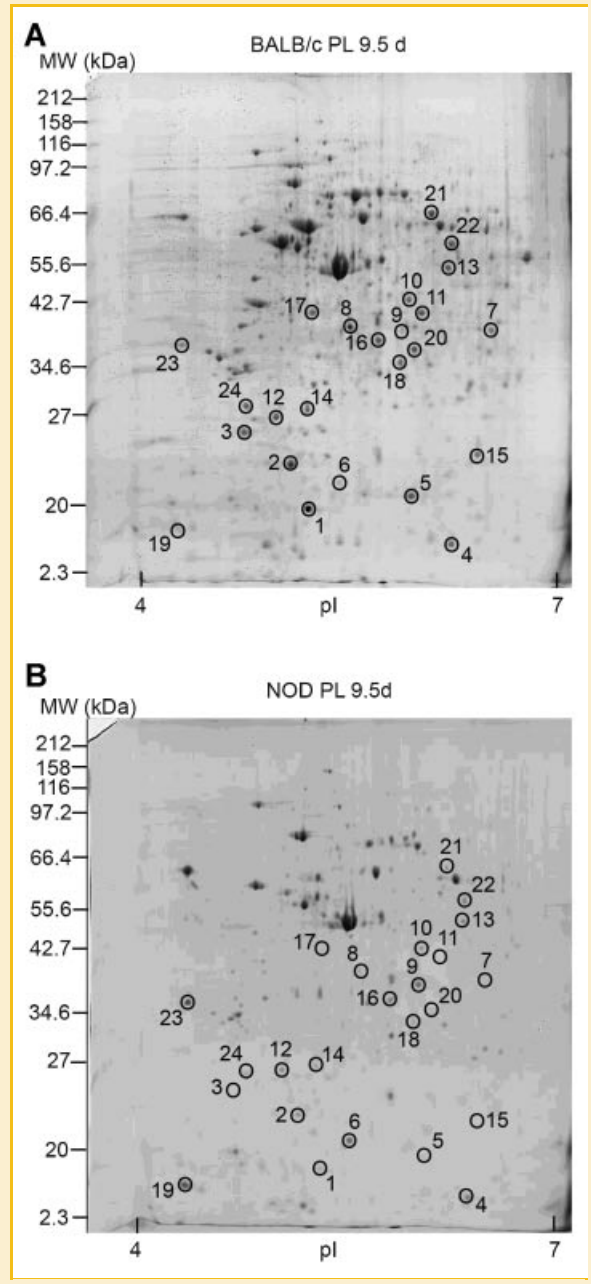

Fig. 1. Two-dimensional electrophoretic map of uterine lymphocyte lysates. Pooled uterine lymphocyte lysates (12 mice per pool) from gestational day 9.5 NOD mice and age-matched non-immunodeficient BALB/c (WT) mice at gestational day 9.5 were used. Representative results of 4 independently conducted experiments. A: From BALB/c mice. B: From NOD mice. The spot number indicates the differentially expressed proteins.

TABLE I. Identified Proteins Possibly Involved in the Mechanism of Pregnancy Tolerance and Embryo Implantation

Spot	Name	Accession no.	MW	pI	Score[a]	Reported functions
1	Eukaryotic translation initiation factor 5A	gi|56800106	16,292	4.85	444	Modulates protein synthesis; neuronal growth and survival; apoptosis; cell proliferation [Huang et al., 2007; Kang et al., 2007]
2	Peroxiredoxin-2	gi|2499469	21,765	5.20	395	Involved in redox regulation of the cell [Lim et al., 1998; Carninci et al., 2005]
3	Tumor protein, translationally controlled 1 (TPT1 or TCTP)	gi|6678437	19,450	5.20	287	Calcium binding; involved in cell proliferation and survival; antiapoptotic activity; chaperone and protein turnover regulator [Miyara et al., 2006; Chen et al., 2007]
4	Fatty acid binding protein 5	gi|6754450	15,127	6.14	175	Primary protection against the cellular toxicity of cholesterol, free fatty acids, and/or lipid oxidants [Campbell et al., 1998]
5	Stathmin 1	gi|9789995	17,264	5.76	284	Regulates microtubule dynamics during cell-cycle progression; plays specific roles during oocyte and preimplantation embryo development [Yoshie et al., 2006]
6	Myeloid bactenecin (F1)	gi|2071985	19,320	5.95	333	Bactenecins are highly cationic polypeptides of neutrophil granules and exert in vitro a potent antimicrobial activity [Sykes et al., 2003; Even-Ram et al., 2007]
7	Cytosolic malate dehydrogenase	gi|74224797	36,487	6.47	291	An essential factor for oocyte maturation and embryo development in mouse; a marker to evaluate metabolic conditions and immunity [Yoon et al., 2006]
8	Capping protein α1	gi|595917	32,731	5.34	512	Important for actin assembly and cell motility [Hart et al., 1997]
9	Capping protein muscle Z-line α2	gi|6671672	32,947	5.69	374	Important for actin assembly and cell motility [Hart et al., 1997]
10	Isocitrate dehydrogenase 3α	gi|18250284	39,613	6.27	174	Regulation of redox metabolism in the mouse oocyte and embryo; prevent the impairment of oxidant factors on oocyte and embryo; placental mitochondrial metabolism [García-Pérez et al., 2002]
11	Acidic ribosomal phosphoprotein P0	gi|13277927	34,165	5.91	324	An essential component of the eukaryotic ribosomal stalk; Its overex pression may cause tumorigenesis [Akamine et al., 2007]
12	Rho GDP dissociation inhibitor β	gi|33563236	22,837	4.97	183	Rho GDIα and Rho GDIβ play synergistic roles in lymphocyte migration and development by modulating activation cycle of the Rho proteins in a lymphoid organ-specific manner; regulates placental extravillous trophoblast cell migration [Ishizaki et al., 2006]
13	Serine (or cysteine) proteinase inhibitor, clade B	gi|114158675	42,548	5.85	473	Its deficiency may result in an acute inflammatory response [Zhang et al., 2007]
14	Rho GDP dissociation inhibitor α	gi|31982030	23,393	5.12	346	Rho GDIα and Rho GDIβ play synergistic roles in lymphocyte migration and development by modulating activation cycle of the Rho proteins in a lymphoid organ-specific manner [Ishizaki et al., 2006]
15	Glutathione peroxidase 1	gi|84871986	22,316	6.74	77	Influence embryo quality and reproductive outcome [Esposito et al., 2000]
16	Pyruvate dehydrogenase (lipoamide) β	gi|18152793	38,912	6.41	229	Participate in pyruvate metabolism and plays a critical role in early embryo development [Dumollard et al., 2007]
17	Chloride intracellular channel 1	gi|15617203	26,996	5.18	172	Regulates the movement of a major cellular anion; involved in fundamental processes that are critical for cell viability; regulate intracellular amino acid concentration [Sonoda et al., 2003; Money et al., 2007]
18	Capping protein (actin filament) muscle Z-line, β isoform	gi|83649737	31,326	5.47	282	The intracellular distribution and migration of many protein complexes and organelles is regulated by the dynamics of the actin filament. Capping protein participates in the modulation of actin filament capping and control the stability of actin filaments [Disanza et al., 2006; Kuhn et al., 2007]
19	Myosin, light polypeptide 6, alkali, smooth muscle and non-muscle	gi|33620739	16,950	4.46	321	It has functions in cell contractility, cell rounding, cell migration, cytokinesis and locomotion; It is concluded that embryonic non-muscle myosin is organized in specific patterns depending on the state of differentiation [Sandquist et al., 2006; Even-Ram et al., 2007]
20	Proteasome (prosome, macropain) 28 subunit α	gi|6755212	28,655	5.73	271	Proteasomes play an important role in protein turnover in living cells. The inhibition of proteasomes affects cell cycle processes and induces apoptosis; participate in proteolysis [Liu et al., 2006]
21	Chaperonin subunit 5	gi|6671702	59,586	5.72	161	Fundamental importance in cell metabolism [Saschenbrecker et al., 2007]
22	Protein disulfide isomerase (PDI) associated 3	gi|112293264	56,643	5.88	149	To protect placental functions against oxidative stress caused by pre-eclampsia; The predominance of PDI in extravillous trophoblast is possibly associated with an increased ability to synthesize collagen or other enzymatic reactions [Shibata et al., 2001; Vilahur et al., 2007]
23	Complement component 1q	gi|112181167	31,006	4.77	259	Augments the presentation of captured antigen by antigen presenting cells to $CD8^+$ T cells; binds to immune complexes to elicit complement-dependent microbial killing; The mouse uterine secretion at the preimplantation stage contains a functionally active complement system [Lu et al., 2007; van Montfoort et al., 2007]
24	Heterochromatin protein 1 homolog β	gi|94390622	15,971	4.97	131	Reduced expression is correlated with increased invasive activity in tumor cells; The modulation of trophoblast cell invasive activity that permits a proper infiltration of these cells into uterine tissue [García-Cao et al., 2004; Nishimura et al., 2006]

[a]Protein scores greater than 63 are significant ($P < 0.05$).

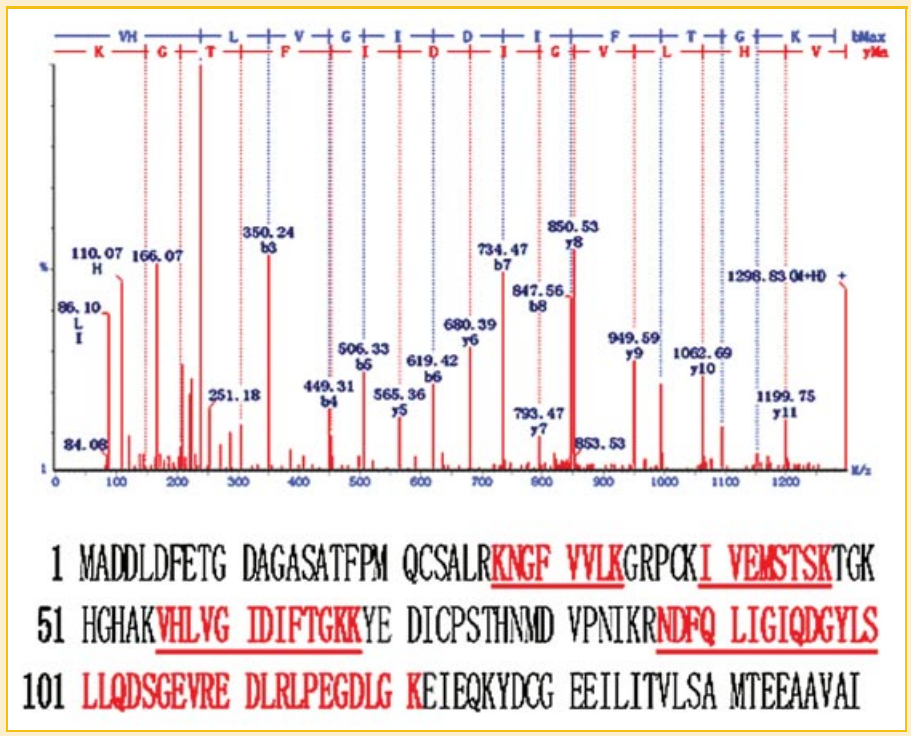

Fig. 2. Mass spectra of MALDI TOF/TOF MS/MS of spot 1 identified as eIF5a. MS/MS signals were derived from the parent ion at m/z 1298.74, for which the amino acid sequence, VHLVGIDIFTGKK, was deduced based upon these b-ions and y-ions in tandem MS spectrum. MS/MS spectrum of eIF5a (m/z 1298.74) is shown. Peptide with m/z 1298.74 was identified as VHLVGIDIFTGKK by MALDI TOF/TOF MS/MS and matched with residues 56–67 of eIF5a. Protein sequence of eIF5a is shown, and matched MS/MS fragmentation is underlined. The PMF and the MS/MS maps of eIF5a were combined to confirm the MS results. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Comparisons of lysates in WT mice showed that 19/24 proteins were expressed at a significantly lower level in splenic lymphocyte lysates than in uterine lymphocyte lysates ($P < 0.01$). These results suggest that these 19 proteins were expressed at a higher level in the pregnant uterus and may play critical functions in the maintenance of allogeneic pregnancy tolerance. In contrast, only 8/24 proteins were expressed at a lower level in splenic lymphocyte lysates than in uterine lymphocyte lysates from NOD mice ($P < 0.01$). These results

TABLE II. The Differential Expression Analysis of Protein in NOD PL, BALB/c PL, NOD SP, and BALB/c SP

Spot	Name	Expression in NOD PL vs. BALB/c PL	Expression in NOD SP vs. BALB/c SP	Expression in BALB/c SP vs. BALB/c PL	Expression in NOD SP vs. NOD PL
1	Eukaryotic translation initiation factor 5A	0.04 ± 0.01	0.94 ± 0.04	0.36 ± 0.04	2.10 ± 0.07
2	Peroxiredoxin-2	0.37 ± 0.03	2.19 ± 0.08	0.27 ± 0.04	0.96 ± 0.07
3	Tumor protein, translationally controlled 1 (TPT1 or TCTP)	0.02 ± 0.01	0.01 ± 0.00	0.32 ± 0.05	2.76 ± 0.13
4	Fatty acid binding protein 5	0.47 ± 0.02	1.82 ± 0.13	0.27 ± 0.05	0.83 ± 0.06
5	Stathmin 1	0.27 ± 0.03	2.5 ± 0.19	0.29 ± 0.05	1.06 ± 0.08
6	Myeloid bactenecin (F1)	3.02 ± 0.13	3.23 ± 0.19	0.55 ± 0.04	0.80 ± 0.08
7	Cytosolic malate dehydrogenase	0.44 ± 0.04	0.88 ± 0.08	0.46 ± 0.05	1.48 ± 0.10
8	Capping protein α1	0.35 ± 0.04	1.58 ± 0.05	0.68 ± 0.04	3.16 ± 0.08
9	Capping protein muscle Z-line α2	3.17 ± 0.12	0.84 ± 0.08	1.73 ± 0.06	0.56 ± 0.07
10	Isocitrate dehydrogenase 3α	0.38 ± 0.02	1.85 ± 0.06	0.46 ± 0.05	1.34 ± 0.11
11	Acidic ribosomal phosphoprotein P0	0.28 ± 0.03	1.17 ± 0.08	0.38 ± 0.07	3.75 ± 0.16
12	Rho GDP dissociation inhibitor β	0.34 ± 0.03	2.71 ± 0.09	2.09 ± 0.08	4.04 ± 0.12
13	Serine (or cysteine) proteinase inhibitor, clade B	0.42 ± 0.02	0.97 ± 0.06	0.27 ± 0.06	0.86 ± 0.07
14	Rho GDP dissociation inhibitor α	0.35 ± 0.04	0.95 ± 0.08	1.62 ± 0.05	2.87 ± 0.11
15	Glutathione peroxidase 1	0.17 ± 0.04	1.14 ± 0.08	0.33 ± 0.05	1.28 ± 0.11
16	Pyruvate dehydrogenase (lipoa mide) β	0.35 ± 0.05	0.47 ± 0.02	0.66 ± 0.05	1.84 ± 0.13
17	Chloride intracellular channel 1	0.17 ± 0.03	0.84 ± 0.04	0.29 ± 0.06	1.11 ± 0.09
18	Capping protein (actin filament) muscle Z-line, β isoform	0.41 ± 0.03	0.91 ± 0.04	1.42 ± 0.05	2.54 ± 0.11
19	Myosin, light polypeptide 6, alkali, smooth muscle and non-muscle	3.73 ± 0.17	2.04 ± 0.05	1.25 ± 0.09	0.43 ± 0.05
20	Proteasome (prosome, macropain) 28 subunit α	0.26 ± 0.04	1.62 ± 0.07	0.74 ± 0.07	3.23 ± 0.09
21	Chaperonin subunit 5	0.27 ± 0.03	0.87 ± 0.03	0.66 ± 0.05	3.09 ± 0.08
22	Protein disulfide isomerase (PDI) associated 3	0.34 ± 0.04	0.67 ± 0.04	0.57 ± 0.05	1.83 ± 0.07
23	Complement component 1q	2.82 ± 0.06	2.09 ± 0.06	0.84 ± 0.07	0.59 ± 0.06
24	Heterochromatin protein 1 homolog β	0.27 ± 0.03	0.94 ± 0.08	0.24 ± 0.08	0.94 ± 0.06

suggest that some of these proteins were not adequately expressed in the pregnant uterus in NOD mice, and this may be correlated with the poor pregnancy outcomes in these mice (Table II).

VERIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS BY WESTERN BLOT ANALYSIS

To confirm the expression levels of the differential proteins identified by a proteomic approach, Western blotting was performed to detect expression of 10 identified candidate proteins (eIF5a, FABP5, GP1, HP1β, PA28α, PRX II, Rho GDI, stathmin, C1q, and MLC2) in NOD and WT lysates. Consistent with the 2-DE and colloidal coomassie blue-staining results, the expression of eIF5a, FABP5, GP1, HP1β, PA28α, PRX II, Rho GDI, and stathmin were significantly down-regulated in NOD uterine lysates, whereas C1q and MLC2 were significantly up-regulated ($P < 0.01$) (Fig. 3). These results demonstrate that proteomic analysis of gestational day 9.5 NOD and WT mice is convincing and indicate that these proteins may play important roles in the modulation of embryo implantation and other important functions during pregnancy.

$CD3^+$ AND $CD49B^+$ PERCENTAGES IN PURIFIED LYMPHOCYTES

The $CD3^+$ and $CD49b^+$ percentages in pooled placenta and decidua basalis lymphocytes were analyzed in both allogeneic pregnant NOD and WT mice.

The $CD3^+$ percentage in WT placenta $CD45^+$ cells was $20.8 \pm 3.6\%$, slightly lower than that in NOD mice ($26.5 \pm 2.7\%$; $n = 4$ per group; $P < 0.05$) (Fig. 4). The $CD49b^+$ percentage in WT placenta $CD45^+$ cells was $74.1 \pm 5.4\%$, markedly higher than that in NOD mice ($51.3 \pm 5.0\%$; $n = 4$ per group; $P < 0.01$) (Fig. 4).

DISCUSSION

Host responses to inflammatory processes depend on innate immunity mechanisms that recognize and respond to the presence of a pathogen in the acute phase, followed by adaptive immunity mediated by clonal selection of specific lymphocytes and leading to long-term protection from diseases [Howard et al., 2004]. During pregnancy, suppression of adaptive immunity may activate the maternal innate immune system [Sacks et al., 1999], increasing stimulation of some leukocyte subsets from the first trimester onward and playing an important role in overall maternal immune defense [Wang et al., 2007]. Excessive innate activation, however, can lead to more severe clinical presentations of some infections and lead to embryo loss during pregnancy.

In our current study, 24 differentially expressed proteins were analyzed in lymphocytes isolated from pregnant uteri and spleens in WT BALB/c and NOD mice. Among these 24 proteins, 20 were found to be highly expressed in uterine lymphocytes isolated from WT mice as compared with NK cell-deficient NOD mice. In contrast, 4 proteins were expressed at a significantly lower level in WT mice than NOD mice. These results provide new insight into the mechanism of unexplained embryo loss. In humans, during weeks 6–18 of gestation the placenta achieves increasing access to the maternal blood supply by extensive remodeling of maternal spiral arteries through extravillous cytotrophoblast invasion [Red-Horse et al., 2004]. In pre-eclampsia and some cases of unexplained spontaneous abortion, poor placentation occurs when invading trophoblast cells fail to gain full access to the maternal uterine lining. Trophoblast cell signaling to maternal immune cells is weak and fails to stimulate normal immune collaboration and a lower adaptive immune response may result [Wang et al., 2007].

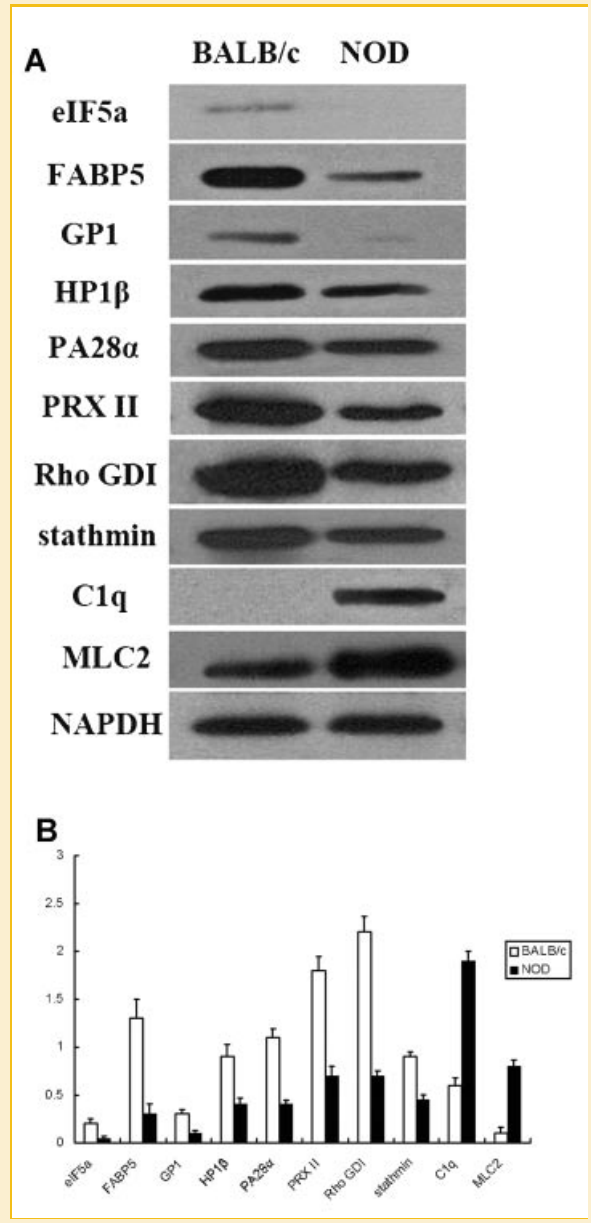

Fig. 3. Representative Western blot analysis. Changes in the expression level of 10 identified candidate proteins (eIF5a, FABP5, GP1, HP1β, PA28α, PRX II, Rho GDI, stathmin, C1q, and MLC2) are shown in uterine lymphocytes isolated from NOD and WT BALB/c mice. A: The expression of eIF5a, FABP5, GP1, HP1β, PA28α, PRX II, Rho GDI, and stathmin were significantly down-regulated, whereas C1q and MLC2 were significantly up-regulated in NOD versus BALB/c mice. NAPDH was used as an internal loading control. B: Histogram shows the relative expression level of the 10 proteins in NOD and BALB/c mice as determined by densitometric analysis ($P < 0.01$ for all). $n = 4$ for each group.

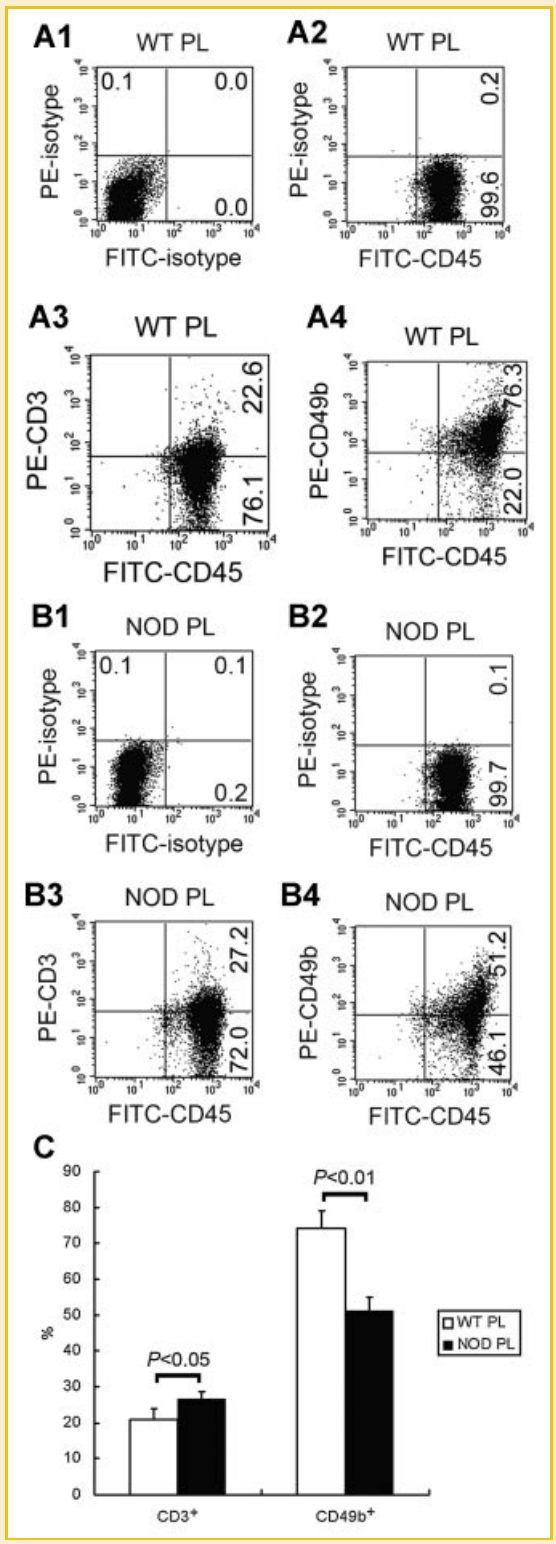

Fig. 4. Analysis of $CD3^+$ and $CD49b^+$ cells in splenic and uterine lymphocytes. A1–A4: WT PL, uterine (pooled placentas together with decidua basalis) lymphocytes from BALB/c mice. B1–B4: NOD PL, uterine lymphocytes from NOD mice. A1,B1: Isotype controls. C: Data summary of flow cytometric analysis using uterine lymphocytes. n = 4 for each group.

In this study we identified 20 highly expressed proteins in WT mice. These proteins include translation initiation factors and regulators of redox metabolism, placental growth and embryo development. Possible functions at the maternal–fetal interface of these proteins are discussed below.

eIF5a (spot 1, Fig. 1 and Table I) has been reported to be an initiation factor that controls translation in mammalian cells [Huang et al., 2007; Kang et al., 2007]. In the current study, differential expression of this protein was found in 2-DE and also confirmed by Western blot analysis. In addition, as a representative of the 24 identified proteins, the peptide mass fingerprinting (PMF) and the MS/MS maps of eIF5a could be combined to confirm the MS results (Fig. 2).

Two proteins (spots 2 and 16) have functions in redox metabolism. PRX II (spot 2) has been reported to be involved in redox regulation of the cell [Lim et al., 1998; Carninci et al., 2005]. In contrast, pyruvate dehydrogenase β (spot 16) modulates redox metabolism in the mouse oocyte and modulates early embryo development [Dumollard et al., 2007]. Since they are expressed at a significantly higher level in uterine lymphocytes of WT mice and correlated with better pregnancy outcomes compared to NOD mice, both kinds of proteins may be involved in the modulation of redox metabolism at early stage of pregnancy in pregnant uterus.

Three proteins (spots 4, 10, and 15) may contribute to placental development. FABP5 (spot 4) has been demonstrated to preferentially bind long chain polyunsaturated fatty acids and thus may be responsible for preferential uptake of these fatty acids by the placenta [Campbell et al., 1998]. This function is extremely important for embryo and placenta development. Isocitrate dehydrogenase 3α (spot 10) plays an important role in placental mitochondrial metabolism, which is essential to oxygen and energy uptake of embryo [García-Pérez et al., 2002]. In addition, genetic inactivation of glutathione peroxidase 1 (spot 15) results in growth retardation, presumably due in part to reduced mitochondrial energy production as a product of increased oxidative stress [Esposito et al., 2000].

Stathmin 1 (spot 5) regulates microtubule dynamics during cell cycle progression, and its expression is higher at embryo implantation sites than at uterine segments between implantation sites [Yoshie et al., 2006]. Therefore, this protein may play important roles in the modulation of embryo implantation, cell cycle progression and other critical functions during pregnancy.

Cytosolic malate dehydrogenase (spot 7) is an essential factor for oocyte maturation and embryo development in mouse [Yoon et al., 2006]. Thus, up-regulated expression of this molecule in WT mice may be beneficial for early-stage pregnancy. In contrast, insufficient expression of this molecule in NOD mice may be related with undesirable pregnancy outcomes.

Four proteins (spots 8, 12, 14, and 18) have reported functions in actin assembly and cell movement. Capping protein α1 subunit (spot 8) is important for actin assembly and cell motility [Hart et al., 1997]. Moreover, Rho GDP dissociation inhibitor β (Rho GDI β) (spot 12) is a key determinant of cell movement and actin-dependent cytoskeletal morphogenesis [Ishizaki et al., 2006]. Rho GDI α (spot 14) is another subunit of Rho GDI, and play a role synergistically with Rho GDI β. Spot 18, capping protein β subunit, modulates

actin-filament nucleation, capping, and disassembly [Disanza et al., 2006; Kuhn and Pollard, 2007].

Chloride intracellular channel 1 (spot 17) is a component of a Cl^- channel in early mouse embryos that is highly permeable to amino acids and may regulate intracellular amino acid concentration [Sonoda et al., 2003; Money et al., 2007]. For technical reasons, Western blot analysis of this molecule was not successfully performed in the present study.

Proteasome 28α (spot 20) is a member in proteasome family. Proteasomes play an important role in protein turnover in living cells. The inhibition of proteasomes affects cell cycle processes and induces apoptosis [Liu et al., 2006]. Chaperonin subunit 5 or cell cycle checkpoint protein (spot 21) modulates chaperone activities and plays an important role in cell cycle [Saschenbrecker et al., 2007]. Both proteins expressed at a markedly lower level in allogeneic pregnant NOD model suggest the existence of abnormal cell cycle, protein turnover, and cell apoptosis in these mice.

Heterochromatin protein 1 homolog β (HP1β; spot 24) is associated with telomere elongation [García-Cao et al., 2004]. Decreased HP1β mRNA and protein expression correlates with invasive potential in human carcinoma cell lines [Nishimura et al., 2006]. In placenta, these results imply that HP1β may modulate invasive potential of trophoblast cells [Nishimura et al., 2006]. Insufficiency of HP1β expression in NOD mice may result in abnormal trophoblast cell infiltration and failure of maternal–fetal blood-supply establishment.

Thus, it is noticeable that these 20 proteins play critical roles in the course of embryo implantation and early-stage development. Insufficient production of these proteins in NOD mice may be correlated with the poor pregnancy outcomes. These proteins may also be critical in the control of allogeneic pregnancy tolerance.

Four proteins have a significantly lower level of expression in WT mice compared to NOD mice. These proteins have reported roles in innate immunity, actin assembly, and cell motility.

Bactencin (spot 6) belongs to myeloid differentiation genes [Sykes et al., 2003], which are highly cationic polypeptides of neutrophil granules and exert in vitro a potent antimicrobial activity [Even-Ram et al., 2007]. Over-expression of bactencin means an extra-active status of antimicrobial effect, similar to Th1-type responses, and to some extent may be harmful to early embryo development.

Capping protein α2 (spot 9) is important for actin assembly and cell motility [Hart et al., 1997]. Myosin (spot 19) regulates cell motility and actomyosin-microtubule crosstalk and plays essential roles in the development of pre-implantation mouse embryos [Sandquist et al., 2006; Even-Ram et al., 2007]. Over-expression of these proteins in NOD mice implies the change of functional status in cell motility and early-stage embryo development.

Complement component 1q (C1q) (spot 23) binds to immune complexes to elicit complement-dependent microbial killing and enhanced phagocytosis. Besides this classical role, C1q also modulates apoptotic cells for clearance by phagocytes. C1q deficiency increases susceptibility to microbial infections and is associated with elevated autoimmunity as characterized by increased apoptotic bodies in tissues [Lu et al., 2007]. In addition, it augments the presentation of antigen captured in immune complexes to $CD8^+$ T lymphocytes [van Montfoort et al., 2007]. However, over-expression of C1q in NOD mice suggests that there may be elevated status of phagocytosis and abnormal complement function in these mice. Subsequently, this status may be harmful to pregnancy outcomes.

The insufficient expression of 20 proteins and over-expression of 4 proteins in abortion-prone NOD mice implies that the balance of protein expression is important for successful pregnancy.

Among the expression pattern of the above-mentioned 24 proteins, 10 were successfully detected using Western blot analysis and confirmed to be consistent with the results of 2-DE and mass spectrometry analysis. More proteins were expressed at a lower level in NOD mice than WT mice. These proteins are reported to be important in cell mobility, trophoblast cell infiltration, embryo development and other critical physiological functions. These results may supply a partial molecular rationale for the spontaneous embryo loss and infertility observed in NOD mice [Wang et al., 2009].

Notably, 17/20 proteins that were expressed at a significantly higher level in WT uterine lymphocytes were expressed at a significantly lower level in WT splenic lymphocytes. This result suggests that up-regulation of these proteins in uterine lymphocytes may be specific, and may be the molecular basis of functional difference between splenic lymphocytes and uterine lymphocytes during pregnancy.

In a recent article we reported that the $Foxp3^+$ percentage was significantly higher in MACS-purified $CD3^+CD25^+$ T cells in allogeneic pregnant BALB/c mice compared to NOD mice [Lin et al., 2009]. In the MACS-purified $CD4^+$ T cells, the percentage of $CD3^+CD4^+CD25^+$ cells in the $CD3^+CD4^+$ cell population was significantly higher in WT mice than in NOD mice at gestational day 9.5. Our results suggest that the constitutional ratio of certain lymphocyte subsets may differ between the isolated uterine lymphocytes from WT and NOD mice during allogeneic pregnancy, and this may exert an influence on the diversity of protein expression displayed by 2-DE in our current study.

Due to these results, we investigated the constitutional ratio of $CD3^+$ and $CD49b^+$ cells in isolated uterine lymphocytes from both strains of mice by flow cytometry. CD45 was used as a common marker of leukocytes. We found than the $CD3^+$ percentage in WT placental $CD45^+$ cell population was slightly lower than that in corresponding NOD groups, whereas the $CD49b^+$ percentage in WT uterine $CD45^+$ cell population was significantly higher than that in corresponding NOD groups. This may partially contribute to the difference in protein expression levels as detected by 2-DE. However, although the expression levels of 24 proteins in WT uterine lymphocytes were at least two fold higher or lower than in the NOD group, the extent of difference in the percentages of $CD3^+$ and $CD49b^+$ lymphocytes isolated from pregnant uteri was much lower. Thus, the difference in protein expression levels cannot be absolutely attributed to the differences in constitutional ratios of $CD3^+$ and $CD49b^+$ cell subsets in lymphocytes.

Restricted by the amounts of proteins isolated from each mouse, 2-DE analysis and other examination could not be performed individually. Under such a condition, samples from several mice were pooled. To our knowledge, this strategy is commonly accepted

in proteomic researches [Cheng et al., 2008; Karp and Lilley, 2009]. Based on the comparison of NOD and WT models, future research can identify the molecules that play critical roles in the modulation of embryo implantation, placenta development and allogeneic pregnancy tolerance, and understand the detailed molecular mechanisms of the prevalence of spontaneous abortion and infertility in NOD mice [Wang et al., 2009].

In summary, the present study outlines the status of differential protein expression in NOD mice and WT mice using uterine and splenic lymphocytes. The identified proteins that are insufficiently expressed in uterine lymphocytes of NOD mice may play critical roles at earlier stage of pregnancy. Aberrant expression of proteins in NOD mice may be correlated with poor pregnancy outcomes.

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