Comparative Study of Protein Profile during Development of Mouse Placenta

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ABSTRACT

To examine the differential protein expression pattern in the 11.5 day post-coitus (dpc) and 18.5 dpc placenta of mouse, we have used the global proteomics approach by 2-D gel electrophoresis (2-DE) and MALDI-TOF-MS. The differential protein patterns of 3 placentae at the 11.5 dpc and 18.5 dpc from nature mating mice were analyzed. Proteins within isoelectric point range of 3.0~10.0, separately were analyzed in 2DE with 3 replications of each sample. A total of approximately 1,600 spots were detected in placental 2-D gel stained with Coomassie-blue. In the comparison of 11.5 dpc and 18.5 dpc placentae, a total of 108 spots were identified as differentially expressed proteins, of which 51 spots were up-regulated proteins such as alpha-fetoprotein, mKIAA0635 protein and transferrin, annexin A5, while 48 spots were down-regulated proteins such as Pre-B-cell colony-enhancing factor 1(PBEF), aldolase 1, A isoform, while 4 spots were 11.5 dpc specific proteins such as chaperonin and Acidic ribosomal phosphoprotein P0, while 3 spots were 18.5 dpc specific proteins such as aldo-keto reductase family 1, member B7 and CAST1/ERC2 splicing variant-1. Most identified proteins in this analysis appeared to be related with catabolism, cell growth, metabolism and regulation. Our results revealed composite profiles of key proteins involved in mouse placenta during pregnancy.

(Key words : Placenta, 2-D gel electrophoresis, Mass spectrometry)

INTRODUCTION

The placenta is a pregnancy specific mammalian reproductive organ responsible for fetal respiration, nutrition and waste removal. Growth and function of the placenta are accurately regulated and coordinated to ensure the exchange of nutrients and waste products between the maternal and fetal circulatory systems at maximal efficiency (Wooding *et al.*, 1994). Successful placental development is important for the maturation, accurate growth and survival of the fetus. Placenta cell lineages develop form trophectoderm cells (Cross, 2005). The primary trophoblast giant cells differentiate from mural trophectoderm, while the polar trophectoderm gives rise to the ectoplacental cone and the extraembryonic ectoderm. In mice the extraembryonic ectoderm forms the chorion that fuses with the allantois, and outgrowth of extraembryonic mesoderm, at around embryonic 8 dpc to form the labyrinthine layer of the placenta. After implantation, the number of trophoblast giant cells increases to over 400 over the next several days (Scott *et al.*, 2000). The process of secondary giant cell differentiation is thought to mediate by cells of the ectoplacental cone (Cross *et al.*, 1994; 2000; 2003; Tanaka *et al.*, 1998; Rossant *et al.*, 2001; Uy *et al.*, 2002; Hemberger *et al.*, 2004; Hughes *et al.*, 2004). The spongiotrophoblast layer of the mouse placenta is derived from ectoplacental precursor cells, and forms on the middle of the placenta. In addition to the primary trophoblast cells developed from the mural trophectoderm, secondary trophoblast giant cells are derived from the spongiontrophoblast. Its function probably has a structural role and also produces several layer-specific secreted factors including anti-angiogenic factors that may prevent the growth of maternal blood vessels into the fetal placenta (Cross *et al.*, 2002). Several studies have reported the specific genes involved in their differentiation and placental cell lineage (Cross *et al.*, 2005; Simmons *et al.*, 2005).

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However, the regulation of placenta growth is not fully understood.

A number of genes have been identified in placental developmental partially by the finding of various lethal embryonic null mutants and placental failure (Yamamoto *et al.*, 1998, Barak *et al.*, 1999; Schorpp-Kistner *et al.*, 1999; Adams *et al.*, 2000; Schreiber *et al.*, 2000; Cross *et al.*, 2003). Recently the gene expression patterns in the developing mouse placenta between 10.5 dpc and 17.5 dpc have been studied by DNA microarray technology (Gheorghe *et al.*, 2006). However, much more research is still need to clear genome-wide gene expressions involved in placental development. The genes expressed in placenta may be expressed with developmentally regulated patterns and the number of mRNA copies may not constantly reflect the number of functional proteins present (Celis *et al.*, 2000).

Currently, 2D gel electrophoresis (2DE) in collaboration of MALDI-TOF mass spectrometry is a frequently applied technique to isolate the composite cellular proteomes (Görg *et al.*, 2000). It gives information about the post-translational modification and expression level of gene products in the normal cells with parameters resulting from the influence of environment factors. By now, the proteomics approach has not been applied to examine the gene expression patterns in the developing mouse placenta. In this experiment, differential protein patterns between 18.5 dpc and 11.5 dpc of mouse placentae was investigated using proteomics approach exploited by utilizing 2DE and MALDI-TOF MS to identify placental proteins regulated with the developing mouse placenta.

MATERIALS AND METHODS

Placental Samples

The two type placentae were obtained from three 11.5 dpc placental and three 18.5 dpc ICR female mice. Placental samples were isolated from uterus on 11.5 day and 18.5 day after the natural mating and stored in liquid nitrogen after collection until use.

Extraction of Solubilized Proteins from Placenta

For 2-D PAGE, soluble proteins were extracted as formerly described (Joubert-Caron *et al.*, 2000), with some modifications by the authors. Briefly, lysis buffer A (containing 1% SDS, 1 mM PMSF, protease inhibitor cocktail (Roche, Basel, Switzerland), 100 mM Tris-HCl, pH 7.0) for pH 3~10 was applied to mouse placenta with equal volume to tissue weight (ex. 100 μl buffer / 100 mg wet weight) (Joubert-Caron *et al.*, 2000), and samples were suspended with sonication for 15 sec, and then chilled in ice. About 800 μl 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 applied to the suspended samples. The samples were shaken gently for 1 hr at room temperature followed by centrifuged at 15,000 rpm for 20 min. The solubilized protein extracts were quantified by Bradford protein assay (Bio-Rad, Hercules, CA, USA) and then final protein samples were stored at −70℃.

2-D Gel Electrophoresis

Precast 18 cm IPG strips with pH 3~10 range were obtained from Amersham Biosciences. One mg of preparative protein samples was used for isoelectric focusing (IEF). The samples was mixed with modified rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2.5% DTT, 10% isopropanol, 5% glycerol, 2% v/v IPG buffer pH 3~10) to total volume of 450 μl (Görg *et al.*, 2000). A mixture of samples was loaded onto an IPG strips (pH 3~10; 180 × 3 × 0.5 mm). The strip was allowed to rehydrate overnight in swelling tray. After rehydration, first dimension, IEF, was performed using an Amersham Pharmacia Multiphor II IEF unit. Automatic isoelectric focusing was carried out for with 1.5×10^5 Vh. Voltage started at 100 V and gradually increased to a final voltage of 8,000 V. After the first dimensional IEF, IPG gel strip 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. The second dimensional separation was performed on 8~16% linear gradient SDS polyacrylamide gels. The gels were placed into 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 per gel until the bromophenol blue marker dye had disappeared at the bottom of the gel.

Staining and Image Analysis of 2-D-Gels

After 2-DE, gels were stained with colloidal coomassie brilliant blue (CBB) G-250. The gels were fixed for 1 hr in fixation solution (30% v/v methanol, 10% v/v acetic acid) and stained the gel with colloidal CBB G250 for 24 hr followed by being destained with 1% acetic acid. The gels were analyzed by Melanie III software (Swiss Institute for Bioinformatics, Geneva, Switzerland). The calculations were applied to the percent volume parameter representative of the protein expression. Variations in abundance were calculated as the ratio of average values (% vol) between the two classes. The process for validation of variant proteins was carried out by human operators (Naïma *et al.*, 2002).

Sample Preparation for MALDI-TOF Mass Spectrometry Analysis

In-gel digestion was performed mainly as previously described (Shevchenko *et al.*, 1996) with some modifications. For coomassie-stained proteins, the gel slab was destained by using 120 μl wash solution (50% v/v acetonitrile, 25 mM NH_4HCO_3, pH 7.8). Then the gel pieces were dehydrated with 50 μl of acetonitrile and dried of for 30 min with a vacuum centrifuge. The dried gel pieces were rehydrated with 5 μl of 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 completion of the digestion, the supernatant was transferred to another Eppendorf tube. To extract residual peptides, the gel pieces were sonicated for 20 min at 30℃ in a solution of 50% acetonitrile/0.5% TFA. Extracted peptides were used for MALDI-TOF analysis. 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 extracted peptide solution from each gel spot piece and the same volume of matrix solution (10 mg/ml α -ciano-4-hydroxycinnamic acid, 0.1% v/v TFA, and 50% v/v acetonitrile) were loaded onto a MALDI sample plate (96 well) and crystallized. For each sample, the average of 500 spectra was obtained and scans were performed twice. 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) and spectra were saved as peak table files (*.pkt) to search against non-redundant protein sequence database on the internet (SWISS-PROT and/or NCBInr [2004/05/01, Data Bank]).

RESULTS

Analysis of Placenta Proteomes by 2-DE

Differential protein expressions in the placentae between 11.5 dpc and 18.5 dpc placentae were evaluated using 2-DE analysis of total protein extracts. The protein expression patterns of mouse placentae obtained at 11.5 day and 18.5 day after mating were compared. For comparison, the placental protein samples were extracted in the same condition. The 2-D gel images obtained from 2-DE of each 1 mg protein sample from 11.5 dpc placenta and 18.5 dpc placenta were shown in Fig. 1. In 2-DE of mouse placental tissue, a large number of proteins were separated across the entire pI/Mw range of the gel. With broad-range analyses using pH 3~10 strip 8~16% gradients gel separation after in the imagemaster 5.0 software following the staining with Comassie blue, approximately 1,500~1,700 protein spots were detected per gels. The spots in Fig. 1A and 1B were indicated as follows; 11S- for 11.5 dpc specific spots, 18S- for 18.5 dpc spe-

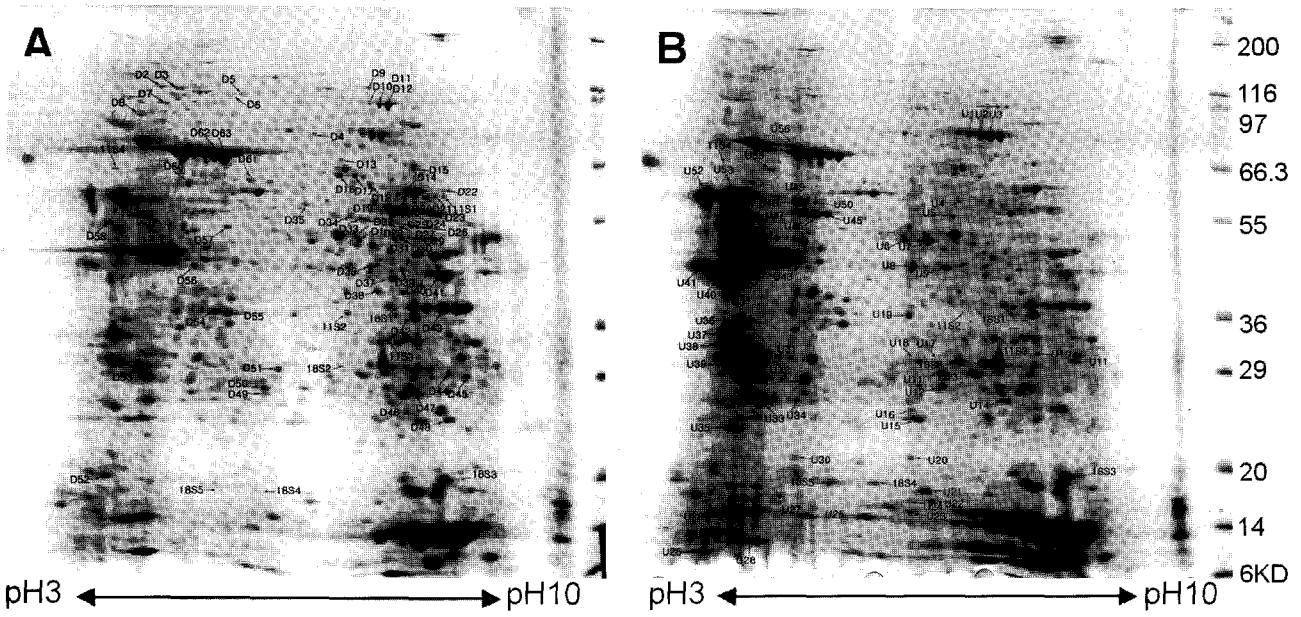

Fig. 1. 2-D PAGE protein separation of 11.5 dpc and 18.5 dpc placentae as visualized by CBB staining. First dimension was in 18 cm 3~10 IPG, and second dimension was in an 8~16% gradient gel. A mean of spots were enumerated with imagemaster 5.0 software. (A) 11.5 dpc, (B) 18.5 dpc.

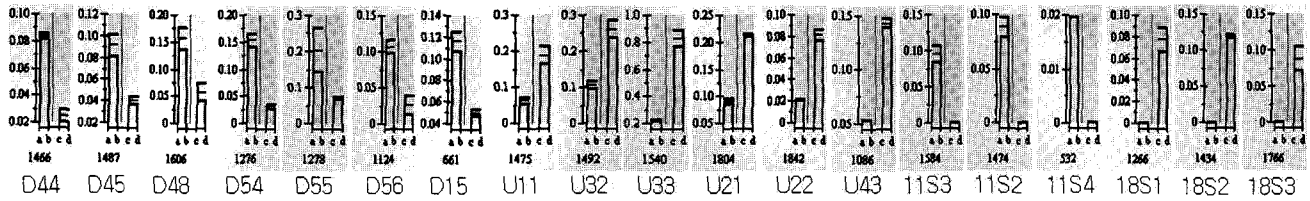

Fig. 2. Differential expression of proteins is % vol histograms. 11S, 11.5 dpc specific spots; 18S, 18.5 dpc specific spots; D, 18.5 dpc down-regulated spots; U, 18.5 dpc up-regulated spots.

cific spots, D- for 18.5 dpc down-regulation spots and U- for 18.5 dpc up-regulation spots. The expression levels of variant protein spots were statistically analyzed and represented by the mean of % volume histograms (Fig. 2). The differentially expressed protein spots between the 11.5 dpc and 18.5 dpc placentae were shown in Fig 3. In the comparison of 11.5 and 18.5 placenta, a total of 108 spots were identified as differentially expressed proteins, of which 51 spots were up-regulated, 48 spots were down-regulated, 4 spots were 11.5 dpc specific and 5 spots were 18.5 dpc specific.

Identification of Differentially Expressed Spots

To identity proteins of differentially expressed spots, unprocessed spectra of samples after tryptic digestion followed by MALDI-TOF MS were processed by DataExplorer™ software and resulted to monoisotopic peaks (Fig. 4). The tryptic peptide masses were used to identify protein candidates in web-based searching software, ProFound (http://129.85.19.192/ profoundbin/ WebProFound.exe). Out of a total 108 spots, 64 spots were used for protein identification and 62 spots were identified as the known proteins from SWISS-PROT and NCBInr database searching. The search results were evaluated on the source of accepted standard that take account into the number of peptides matched to the candidate protein, the coverage of the candidate protein's sequence by the matching peptides and agreement of the experimental and theoretical pI and Mw with values. Proteins identified were presentation in Table 1. In the comparison of 11.5 dpc and 18.5 dpc placental samples, a total of 62 spots were identified as differentially expressed proteins, of which 24 spots were up-regulated proteins such as alpha-fetoprotein, mKIAA0635 protein, transferring and annexin A5, while 31 spots were down-regulated proteins such as Pre-B-cell colony-enhancing factor 1(PBEF), aldolase 1 and A isoform. And 4 spots were 11.5 dpc specific proteins such as chaperonin and acidic ribosomal phosphoprotein P0, while 3 spots were 18.5 dpc specific proteins such as aldo-keto reductase family 1, member B7 and CAST1/ERC2 splicing variant-1. However, 2 spots could not be identified. Proteins related with structural component of chromatin, immune system, re-

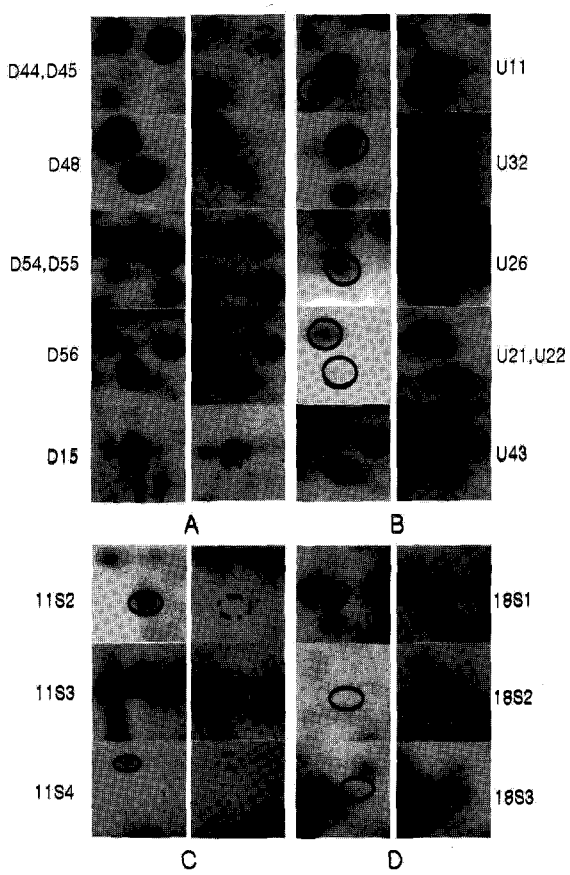

Fig. 3. Differentially expressed protein spots between 11.5 dpc and 18.5 dpc placentae. A black dotted line represents down-regulation and solid line represents up-regulation. (A: 18.5 dpc down-regulated spots, B: 18.5 dpc up-regulated spots, C: 11.5 dpc specific spots, D: 18.5 dpc specific spots,).

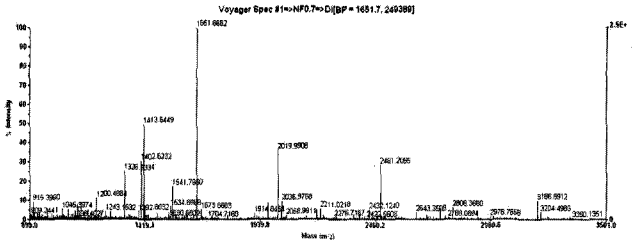

Fig. 4 MALDI-TOF-MS spectra obtained for D15 spots. Database searching allowed the identification of mouse bactenecin 1, as reported in Table 1.

Table 1. Differentially expressed proteins identified by MALDI-TOF

Spot ID	Est'd Z [a)]	Accession No	Protein information	%	pI	kDa
11 dpc specific spots						
11S1	2.21	AAA37418.1	chaperonin	23	8.8	58.74
11S2	1.48	AAH03833.1	Acidic ribosomal phosphoprotein P0	46	5.9	34.38
11S3	2.39	Q9CVB6	Actin-related protein 2/3 complex subunit 2	75	7.1	22.29
11S4	2.33	BAC27092.1	unnamed protein product	44	5.3	60.74
18 dpc specific spots						
18S1	1.78	NP_033861.1	aldo-keto reductase family 1, member B7	53	6.5	36.17
18S2	1.62	AAA37190.1	alpha-fetoprotein	23	5.5	49.2
18S3	1.54	AAR14793.1	CAST1/ERC2 splicing variant-1	15	6.5	112.3
18 dpc down-regulation spots						
D1	2.32	NP_080237	Glycine amidinotransferase, mitochondrial precursor	31	8.4	48.92
D8	2.43	BAB20776.1	heat shock protein 90 beta	44	5	82.37
D10	1.3	NP_598329.1	ribosome binding protein 1 isoform b	16	9.9	72.52
D13	2.33	AAH11301.1	Sdha protein	33	7.2	72.31
D14	2.3	NP_035229.2	pyruvate kinase, muscle	42	7.2	58.54
D15	2.35	AAG28459.1	transketolase	41	6.5	61.28
D16	2.22	NP_058017.1	stress-induced phosphoprotein 1	34	6.4	63.35
D18	1.61	Q9D2N4-2	Dtna Isoform 2 of Dystrobrevin alpha	19	6.3	78.62
D19	2.39	AAH04059.1	Pre-B-cell colony-enhancing factor 1	33	6.7	55.78
D20	1.8	NP_031664.2	chaperonin subunit 7 (eta)	20	8.3	60.26
D25	1.42	AAH04059.1	Pre-B-cell colony-enhancing factor 1	32	6.7	55.78
D27	2.09	AAH04059.1	Pre-B-cell colony-enhancing factor 1	31	6.7	55.78
D34	2.27	AAH52408.1	Fascin homolog 1, actin bundling protein	26	6.4	55.41
D36	1.39	NP_031625	capping protein (actin filament), gelsolin-like, full insert sequence	43	6.5	39.11
D38	2.05	NP_080628	Ppid 40 kDa peptidyl-prolyl cis-trans isomerase	38	7.1	41.22
D39	2.33	CAA30275.1	aspartate aminotransferase	51	6.7	46.57
D40	1.33	NP_031464.1	aldolase 1, A isoform	38	8.8	39.91
D41	1.18	NP_031464.1	aldolase 1, A isoform	42	8.8	39.91
D42	1.73	NP_034829.1	lactate dehydrogenase A	35	7.8	36.91
D43	2.34	NP_032110.1	glyceraldehyde-3-phosphate dehydrogenase	62	8.7	36.15
D44	1.47	NP_620046.1	U7 snRNP-specific Sm-like protein LSM10	50	8.2	13.86
D46	2.08	NP_033417.1	RAN, member RAS oncogene family	39	7	24.62
D47	2.4	NP_033441.1	triosephosphate isomerase 1	57	6.9	27.12
D48	2.03	NP_035164.1	peroxiredoxin 1	50	8.6	22.5
D50	1.43	NP_031479.1	peroxiredoxin 6	41	6	24.95
D51	1.33	AAA37552.1	cytokeratin	40	5.2	47.52
D53	1.45	NP_598557	Rho GDP-dissociation inhibitor 1	41	5.1	23.46
D59	1.32	NP_034607.2	heat shock protein 1 (chaperonin)	37	5.7	61.15
D62	2.21	P07724	albumin	24	5.5	67.54
D63	2.16	P07724	albumin	22	5.5	67.54

Table 1. Continued

spot ID	Est'd Z[a)]	accession No	Protein Information	%	pI	kDa
18dpc up-regulation spots						
U3	1.66	NP_598738.1	transferrin	26	7	79.41
U5	1.41	NP_033786.1	aldehyde dehydrogenase 2, mitochondrial	30	7.7	57.16
U12	1.04	AAA37190.1	alpha-fetoprotein	21	5.5	49.2
U13	1.74	NP_033784.1	albumin	25	5.8	71.26
U18	2.19	AAA37190.1	alpha-fetoprotein	23	5.5	49.2
U19	1.38	AAA37423.1	cytosolic malate dehydrogenase	30	6.2	36.67
U20	2.14	NP_031664	T-complex protein 1 subunit eta	26	8.3	60.27
U21	1.97	1513495A	Cu/Zn superoxide dismutase	43	6	15.96
U25	1.36	NP_034304	Fatty acid-binding protein, heart	43	6.1	14.8
U26	1.32	A2A446	SET domain containing 3	57	9.5	17.56
U27	1.93	NP_032521	Lgals1 Galectin-1	37	5.3	15.27
U28	1.22	NP_035443.1	S100 calcium binding protein A6 (calcyclin)	46	5.3	10.1
U29	1.68	BAC65772.1	mKIAA1302 protein	13	6.8	137.4
U30	1.56	AAA37190.1	alpha-fetoprotein	15	5.5	49.2
U31	1.47	CAA31455.1	gamma-actin	43	5.6	41.43
U32	2.15	AAA37170.1	A-X actin	31	5.2	42.1
U33	2.12	CAC81903.1	albumin	50	5.5	24.39
U34	1.86	1UIO	Chain , Adenosine Deaminase (His 238 Ala Mutant)	37	5.4	39.93
U35	2	NP_035693	Prdx2 Peroxiredoxin-2	33	5.2	21.97
U36	2.28	AAH03716.1	Anxa5 protein	36	4.8	35.79
U37	1.44	P09103	P4hb Protein disulfide-isomerase precursor	18	4.8	57.62
U48	2.01	NP_033784	Serum albumin precursor	21	5.8	71.23
U49	2.36	NP_033784	Serum albumin precursor	26	5.8	71.23

[a)] Z score is the distance to the population mean in unit 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" that the search is a correct identification. (The following is a list for 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]).

versible hydratation of carbon dioxide, signal transduction, signaling cascades of growth factors, molecule transport and protein degradation were differentially expressed between 11.5 dpc and 18.5 dpc placentae.

DISCUSSION

The molecular basis of placental development remains scantily. Recent studies have begun to shed some light on this process, and numerous genes have been identified to be essential for the proper differentiation of placental cell lineages and fetal survival. The present study has identified several subsets of proteins that were differentially regulated during placental development. We analyzed a proteomics study in which we compare 11.5 dpc and 18.5 dpc mouse placentae because pregnant 11.5 day and 18.5 day in mice are placenta formation mid-phase and last-phase during the 20 days of gestation and placentation evolves from an initial choriovitelline pattern to a cho-rioallantoic pattern around 11.5 days (Malassiné *et al.*, 2003). The

expression levels of 108 spots differed significantly when placenta proteins on 11.5 dpc and 18.5 dpc were compared. The threshold, at least 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. Earlier investigation reported that Pre- B-cell colony- enhancing factor appeared to be at the proximal end of the pathway to labor initiation and may link sterile distention-induced labor with that of infection-induced labor (Ognjanovic *et al.*, 2003). In our experiment, expression of protein Pre- B-cell colony-enhancing factor 1 in pregnancy on 11.5 dpc was higher than the pregnancy last stage 18.5 dpc expression. The spot U3 up-regulated on 18.5 dpc was identified as transferrin, whose homozygous mutant mice exhibited to be not viable beyond embryonic 12.5 dpc and had severe anemia with hydrops as well as diffuse neurologic abnormalities (Levy *et al.*, 1999). And 18.5 dpc specific spots (18S2) were identified as alpha-fetoprotein (AFP). Alpha-fetoprotein is a major plasma protein in the fetus, where it is produced by the yolk sac and liver. The AFP has many physiologic functions, among which AFP is known to be in correlation with perinatal distress/pregnancy and has long been used as a biomarker for fetal birth defects (Mizejewski, 2007). Calcium binding protein, ribosomal phosphoprotein and albumin were identified to be up-regulated in 18.5 dpc placenta, which is accordance with recently reported results (Gheorghe *et al.*, 2006). Even though our studies have revealed composite protein expression profile in mouse placenta, further analysis should be performed in order to understand the precise regulation and specific functions of these proteins in placenta.

Proteomic analysis has allowed us to describe protein expression patterns and profiles in the developing mouse placenta. Our results revealed composite profiles of key proteins involved in mouse placenta during pregnancy and demonstrated that several functional class proteins are up and down-regulated at specific placental development. These differently expressed proteins may have significant implications the study of for placental development.

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