Proteome Differences in the First- and Third-Trimester Human Placentas

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Abstract

Placenta is a transient and unique pregnancy tissue that supports the fetus nutritionally and metabolically. Expression of the unique placental proteins in different stages may influence the development of the fetus as well as the pregnancy outcome. The present study aimed to compare the total placental proteome differences between the normal first- and third-trimester human placentas. In the current study, placental proteome was compared between normal first- and third-trimester placentas using 2-dimensional polyacrylamide gel electrophoresis method for separation and matrix-assisted laser desorption/ionization time-of flight mass spectrometry technique for identification of the proteins. Despite the overall similarities, comparison of the mean intensity of the protein spots between the first- and third-trimester placental proteomes revealed that 22 spots were differentially expressed ($P < .05$) among which 11 distinct spots were successfully identified. Of the 11 differentially expressed proteins, 4 were increased (protein disulfide isomerase, tropomyosin 4 isoform 2, enolase 1, and 78-kDa glucose-regulated protein), while the remaining 7 (actin γ 1 propeptide, heat shock protein gp96, α 1-antitrypsin, EF-hand domain family member D1, tubulin α 1, glutathione S-transferase, and vitamin D binding protein) showed decreased expression in the placentas from the first-trimester compared to the full-term ones. In summary, the results of the present study as the first research on the comparison of the first- and third-trimester human placental proteomes introduced a group of 11 proteins with altered expression. Interestingly, some of these proteins are reported to be altered in pregnancy-related disorders.

Keywords

placenta, proteomics, first trimester, third trimester, mass spectrometry

Introduction

Success or failure of a pregnancy depends on formation of a normal placenta. Placenta is a transient structure that supports the fetus nutritionally and metabolically.¹ Moreover, placenta is a dynamic process, and proper migration of the fetal trophoblast cells through endometrium is a key step in the placental formation.² Several different biological and hemostatic processes are involved in the regulation of a normal placenta. Expression of the unique placental proteins in different stages maintains the pregnancy and supports the proper development of the fetus.³ The most important cells of a placenta are trophoblast cells. Without trophoblast cells, a pregnancy could not proceed. Trophoblast proliferation and differentiation must be tightly controlled, and the oxygen tension is one of the major regulators of trophoblast cells during placenta formation.⁴ Although hypoxia stimulates cytotrophoblast proliferation, high oxygen tension promotes cytotrophoblast differentiation.⁴ Since implantation up to the 10th week of gestation, placenta is in low-oxygen environments, which stimulate proliferation of cytotrophoblast and inhibit differentiation and invasion of extravillous trophoblast (EVT) cells.⁵ At the 12th week, O₂ tension will increase until the end of the first

trimester. In this period, oxidative stress will increase within the placenta and EVT cells are more invasive.⁵ From the beginning of the third trimester until term, proliferation of trophoblast cells will decrease, while proliferation of endothelial cell increase.⁵

Although genomics and transcriptomics have been widely used for investigating the possible genes that might be associated with a normal placenta or the pathogenesis of pregnancy-related disorders, really scarce articles have been

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published regarding the human placental proteome. Proteomics refers to the identification and quantification of all proteins derived from the tissue or the whole genome. Proteomics has been considered as a new technique for investigation of the possible proteins associated with placenta and pregnancy-related disorders.^{6,7} Despite the major role of the placenta in pregnancy, little is known about the proteome of this crucial tissue or proteome changes during a normal human placenta or pregnancy-related diseases. Investigation of placental proteome may provide us with more information about the etiology and pathophysiology of the placental-related diseases, such as preeclampsia and miscarriage. Although 2 articles have been published regarding the full-term placental proteome,^{8,9} no studies have been conducted on the first-trimester whole human placental proteome up to now. Moreover, the human placental proteome differences between the first- and third-trimester placentas have not been compared yet. Mine et al published the first human placental proteome map using whole full-term placenta as the protein source in 2007.⁸ Using 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) coupled with Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) technique, they introduced 110 distinct proteins as the first placental proteome map. Furthermore, Mushahary et al published the second human placental proteome map including 117 unique proteins and added a group of new proteins to the previous placental proteome in 2013.⁹ Up to now, only a few proteomic studies have been performed on the placental tissue or cell lines and the relationship between the placental proteome changes and pregnancy-related disorders, such as pregnancy loss and preeclampsia.^{8,10-17} Considering the scarcity of the published studies on the proteome changes during fetus development, the present study aimed to compare the total placental proteome differences between the normal first- and third-trimester human placentas. In order to fulfill this goal, placental proteins from normal first- and third-trimester human placentas were extracted and subjected to 2D-PAGE. After staining, the gels were scanned and spot intensities were determined and compared between the first- and third-trimester placentas.

Materials and Methods

Placental Samples

In this study, 6 human whole normal placental samples in the first and 6 in the third trimester were collected. All the participants had a history of at least 2 previous successful normal pregnancies and were selected from caucasians living in the South-West of Iran after matching their ages (28.8 ± 3.1 and 27.6 ± 2.9 years for first and third trimester, respectively). The normal first-trimester placentas were selected from the late first-trimester pregnant women referring to the legal abortion committee, Shiraz University of Medical Sciences, due to mother indication for abortion (such as heart disease). This group of pregnant women had normal pathologic report and had not used additional medications prior cesarean section.

Besides, the mean gestational age of the first-trimester placentas was 10.8 ± 2.1 weeks. On the other hand, the normal full-term placentas were selected from normotensive pregnant women who underwent an elective-term cesarean section with the gestational age of 38.1 ± 1.1 weeks and a normal pathologic report.

The research protocol was approved by the local ethics committee of the university, and written informed consents for using the placentas in the study were obtained from all the participants. After collecting the placentas, 5 different areas of each placenta (including both maternal and fetal sides) were punched, pooled, and washed in cold normal saline to reduce and eliminate the contaminating blood. All placentas were punched according to the fix pattern of number 5 in a dice. Using a sterile scalpel, 4 areas from 4 directions with 1-cm depth and 1 from the center with 3-cm depth were punched. The weight of each punch was about 500 mg. All the samples were stored in liquid nitrogen until protein extraction.

Protein Extraction

Total protein from the placental tissues was extracted as described previously.¹⁷ The concentration of the extracted proteins was determined using 2D Quant kit (Amersham, Uppsala, Sweden). Then, the extracted proteins were stored at -70°C until performing the proteomics study.

Two-Dimensional PAGE

The first dimension of PAGE was carried out using 3 linear pre-cast 18 cm immobilized pH gradient strips (pH 4-7, 5-8, and 3-10; Bio-Rad, Hercules, California) for each sample. In order to minimize the variation, first- and third-trimester protein extracts were run and stained simultaneously in a twin gel electrophoresis system (SCIE-PLAS, Cambridge, United Kingdom) for the second dimension as described previously.¹⁷

Staining, Statistical Analysis, and Spot Detection

First- and third-trimester 2D gels were stained with Coomassie Brilliant Blue (CBB) or silver nitrate.^{18,19} In addition, 2D Image Scanner and Image Master 2D Platinum software (Pharmacia, Uppsala, Sweden) were used for scanning and spot analyzing, respectively. To avoid variation in analysis, the same parameters were used for each gel. Besides, a single master gel image containing all the spots was prepared in each group as the reference gel. After determining the percentage of intensity (% intensity) for each spot, the mean intensity of each spot was compared by nonparametric Mann-Whitney *U* test using the SPSS software, v 16 (SPSS Inc, Chicago, Illinois). *P* values less than .05 were considered as statistically significant.

After manually excising the differently expressed spots from CBB-stained gels, the MALDI TOF/TOF technique was used for identification of the tryptic protein spots. Mass spectrometry analysis was performed in Sir Henry Wellcome Functional Genomics Facility, University of Glasgow, using 4700 MALDI-TOF/TOF Proteomics Analyzer instrument (Applied

Biosystems, United Kingdom). Besides, MASCOT program search algorithm (<http://www.matrixscience.com>) based on the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov>) was used for database search. One missed cleavage with trypsin and 2 modifications (carbamidomethylation of cysteine and oxidation of methionine) were allowed in the search setting. Statistical confidence limits of 95% were applied for protein. In the present study, MASCOT protein scores greater than 78 were considered as statistically significant ($P < .05$).

Western Blot Analysis

To confirm the mass results, Western blot technique was used for detection of 1 downregulated (vitamin D binding protein [DBP]) and 1 upregulated (Protein disulfide isomerase) protein using appropriate commercial antibodies (ab70415 for Protein disulfide isomerase and ab23484 for DBP; Abcam, AL-Ain, United Arab Emirates) on polyvinylidene difluoride (PVDF) membrane-transferred 2D gels. The 2 mentioned differentially expressed proteins were selected because their location in 2D gels was very close to each other and to check false picking the spots.

For Western blotting of each protein, at first, two 2D gels were simultaneously run in a twin gel electrophoresis system. In the next step, 1 gel was stained with CBB, while the second was transferred on PVDF membrane using a semi-dry transfer system (Amersham). Then, appropriate antibodies were used for blotting of PVDF membrane-transferred protein spots. Anti-mouse Horse radish peroxidase-conjugated antibody (ab97023; Abcam) was used as the secondary antibody, and sigma Fast 3,3'-diaminobenzidine (DAB) tablets (Sigma, Steinheim, Germany) were used for visualization of the blotted spots. After all, the location of each blotted spot was compared with the manually excised spots from the CBB-stained gel. Moreover, the same antibodies were used to confirm the differences in protein disulfide isomerase and DBP expression in the first- compared to the third-trimester placentas. In doing so, 50 μ g of the placental extracted protein (first and third trimesters) were separated by 15% sodium dodecyl sulfate PAGE. After transferring the proteins onto the PVDF membrane, they were blotted with antibodies against protein disulfide isomerase or DBP, while antiglyceraldehyde 3-phosphate dehydrogenase antibody (ab9484; Abcam) was used as housekeeping control protein. The same secondary antibody and DAB tablets were used for visualization of the blotted spots or bands as described in the previous section. The band densities were evaluated to quantify the results.

Results

Identification of the Late First-Trimester Proteome and its Comparison to that of the Full-Term Placentas

At first, 800 protein spots in different pH ranges (3-10, 5-8, and 4-7) were selected from silver-stained gels (of about 1300

detected spots in references gels) in each group (late first or third trimester) by ranking according to the percentage of spot intensities (% intensities). Comparison of the 800 spots between 6 full-term and 6 first-trimester placentas revealed that the placental proteomes were overall the same. Nonetheless, despite the overall similarities, comparison of the percentage of intensities between 6 late first-trimester and 6 full-term placentas revealed that 22 spots were differentially expressed (Mann-Whitney U test, $P < .05$). Among these 22 spots, 11 distinct spots were successfully picked up from CBB-stained gels and characterized using MALDI TOF/TOF technique (Figure 1; Table 1). As indicated in Table 2, 4 of the 11 differentially expressed proteins were upregulated (protein disulfide isomerase, tropomyosin 4 isoform 2, enolase 1, and 78-kDa glucose-regulated protein), while the remaining 7 (actin γ 1 propeptide, heat shock protein gp96, α 1-antitrypsin, EF-hand domain family member D1 (EFHD1), tubulin α 1, glutathione S-transferase, and DBP) showed decreased expression in the normal late first-trimester placentas compared to the normal full-term ones (Table 2).

Validation of Mass Results by Western Blot Techniques

The results of Western blot using commercial antibodies against 2 selected proteins (protein disulfide isomerase and DBP) confirmed the mass results for both protein spots. For each monoclonal antibody, the blotted spot was located in the correct place regarding size and isoelectronic point (pI) compared to the picked corresponding spots. Moreover, as indicated in Figure 2, in line with the results obtained from analysis of spot intensities in the first- and third-trimester 2D-PAGEs, Western blot results also indicated that protein disulfide isomerase was overexpressed, while DBP showed decreased expression in the placentas from the first-trimester compared to the full-term ones ($P < .004$, Mann-Whitney U test).

Discussion

Although a few studies have been conducted on human placental proteome in pregnancy-related disorders, no studies have been performed on the normal first-trimester total placental proteome until now. On the other hand, first- and third-trimester human placental proteomes have not been compared yet. For the first time in the present study, human placental proteome has been compared between the late first- and third-trimester placentas. The results of this study indicated that despite the overall similarity between the late first- and third-trimester placental proteomes, at least 11 distinct proteins were differentially expressed in the late first-trimester placentas compared to the full-term ones.

Considering the gestational age of our first-trimester placentas (12.8 ± 2.1 weeks), it seems that trophoblast cells start to experience a new condition; that is, an environment with more O_2 tension and oxidative stress, compared to few weeks ago. Interestingly, the 4 upregulated proteins were well matched to this placental condition.

Figure 1. Two comparative silver-stained 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) gels (15%, pH 4-7 linear) annotated for differentially expressed spots. A, Full-term normal placenta. B, Normal late first-trimester placenta. C, Magnified view of differentially expressed spots.

Topomyosin 4 (Tm4) is defined as a cytoskeleton protein that is involved in growth and repair/regeneration in response to injury. Due to the invasive nature of EVT cells in this period, the overexpression of Tm4, compared to the third trimester, seems to be a normal physiological response to protect the placenta from the adverse effects of injuries.

In such oxidative stress which is seen in the first-trimester placentas, upregulation of the proteins involved in thiol/disulfide oxidoreductases system, including protein disulfide isomerase, is also accounted as a normal physiological response of the placental oxidative stress. Interestingly, in preeclamptic placentas, which have abnormal placentation and increased oxidative stress, overexpression of the protein disulfide isomerase have been reported.²⁰ Moreover, our previously published data showed that protein disulfide isomerase was upregulated in the preeclamptic placentas.¹⁷ GRP78 is a heat shock protein, which is recently characterized as p53 partner in trophoblast cells.²¹ Arnaudeau et al indicated that GRP78 in interaction with p53, a well-known growth suppressor protein, acted as a regulator of trophoblast invasion, an active process in the first-trimester compared to the third-trimester placentas.²¹ The fourth overexpressed protein is enolase 1,

which is involved in glycolysis, a process which is more active in the first- compared to the third-trimester placentas.²²

In this study, the comparison of late first- and third-trimester placentas revealed that at least 7 proteins were downregulated in the first-trimester placentas (or upregulated in the third trimester ones). In the third trimester, however, proliferation of trophoblast cells decreased, while endothelial cell proliferation increased.⁴ Both actin γ 1 propeptide and tubulin α are cytoskeleton proteins that are involved in endothelial cell proliferation. Therefore, overexpression of actin γ 1 and tubulin α is expected in the endothelial cells of the third trimester.

Heat shock protein GP96 and GST are 2 stress response proteins that showed downregulation in the late first- compare to the third-trimester placentas. This finding indicated that in a normal placentation, third-trimester placentas are more under stressful conditions compared to the first-trimester ones. Thus, overexpression of heat shock protein GP96 and GST in the third-trimester placentas seem to be a normal physiological response to protect the full-term placenta from the adverse effects of stress. Normally, the first-trimester placentas are under stable and less stressful conditions compared to the second- and third-trimester ones.²³ In line with our results, increase in the

Table 1. Differentially Expressed Protein Spots in the Late First-Trimester Placentas Compared to the Full-Term Ones, Identified by MALDI TOF/TOF/Mass Technique.

Spot ^a	Protein Name	Mr ^b	pI ^b	Peptides ^c	Score ^d	Accession No.
1	Actin, γ 1 propeptide	41837	5.31	14	192	gi 4501887
2	Heat shock protein gp96 precursor	90309.1	4.73	23	151	gi 15010550
3	Protein disulfide isomerase	57042.9	6.1	19	134	gi 860986
4	Tropomyosin 4 isoform 2	28618.5	4.67	13	93	gi 4507651
5	α 1-Antitrypsin	46848.1	5.43	12	115	gi 177831
6	EF-hand domain family, member D1	27024.9	5.34	16	176	gi 20149496
7	Tubulin α 1	50547.7	4.96	20	373	gi 14389309
8	Enolase I	47350.4	6.98	12	126	gi 203282367
9	78-kDa glucose-regulated protein	72491.5	5.07	21	482	gi 2506545
10	Glutathione S-transferase	23595.1	5.43	12	499	gi 2204207
11	Vitamin D binding protein	54498.6	5.33	13	228	gi 34785355

Abbreviations: MALDI TOF, Matrix-assisted laser desorption/ionization time-of-flight; Mr, average mass of the protein; pI, isoelectric point.

^aSpot numbers are the same as the spot labels in Figure 1.

^bTheoretical/mass (dalton) or pI.

^cNumber of match peptides.

^dProtein scores of greater than 78 were considered as statistically significant ($P < .05$).

Table 2. Comparison of the Mean Percentage of Intensity of the Differentially Expressed Spots Between the Late First- and Third-Trimester Normal Placentas.

No.	Protein Name	% Intensity, Mean $\pm$ SD			
		First	Third	Change ^a	P Value ^b
1	Actin, γ 1 propeptide	0.194 $\pm$ 0.068	0.380 $\pm$ 0.112	↓	.008
2	Heat shock protein GP96 precursor	0.203 $\pm$ 0.031	0.332 $\pm$ 0.042	↓	.008
3	Protein disulfide isomerase	0.391 $\pm$ 0.087	0.267 $\pm$ 0.019	↑	.008
4	Tropomyosin 4 isoform 2	0.297 $\pm$ 0.053	0.170 $\pm$ 0.058	↑	.032
5	α 1-Antitrypsin	0.240 $\pm$ 0.064	0.335 $\pm$ 0.070	↓	.032
6	EF-hand domain family, member D1	0.133 $\pm$ 0.062	0.245 $\pm$ 0.049	↓	.016
7	Tubulin α 1	0.150 $\pm$ 0.027	0.221 $\pm$ 0.016	↓	.008
8	Enolase I	0.393 $\pm$ 0.054	0.260 $\pm$ 0.062	↑	.016
9	78-kDa glucose-regulated protein	0.193 $\pm$ 0.023	0.108 $\pm$ 0.037	↑	.008
10	Glutathione S-transferase	0.068 $\pm$ 0.034	0.200 $\pm$ 0.037	↓	.008
11	Vitamin D binding protein	0.173 $\pm$ 0.012	0.274 $\pm$ 0.062	↓	.008

Abbreviation: SD, standard deviation.

^aUpregulation or downregulation of the spots in the normal late first-trimester placentas compared to the full-term ones indicated by ↑ and ↓, respectively.

^bExact P values calculated by Mann-Whitney U test.

level of GST during the pregnancy period has been reported before.²⁴ Moreover, glutathione S-transferase-null genotype has been indicated to be correlated with preterm delivery susceptibility in Asians.²⁵

Furthermore, hormones are considered as a main director for a normal placentation. For instance, thyroid hormones have a crucial role in the development of the fetus and placentation.²⁶ The transport of thyroid hormones from the mother to the fetus on one hand and supply of thyroid hormones for the fetus on the other hand require the expression of thyroid hormone binding proteins by the placental tissue. In this respect, 4 thyroid hormone binding proteins, including, α 1-antitrypsin, have been shown to be expressed in the placental tissue.²⁷ Findings of our study indicated that α 1-antitrypsin was upregulated in the third trimester. Interestingly, overexpression of the DBP was also observed in comparison to the first-trimester placentas. The

DBP is a multifunctional protein that is involved in transport of vitamin D to the fetus. Trophoblast cells are also involved in the transport of vitamin D, and the expression of DBP on the surface of human placental trophoblast cells has been reported.²⁸ Vitamin D status may affect the risk of pregnancy-related, third-trimester disorders, such as preeclampsia, through several plausible mechanisms, including regulation of placental invasion and angiogenesis or its immunomodulatory properties.^{29,30} Moreover, the neonatal skeletal development depends on the placental vitamin D status. In fact, third trimester fetuses need more vitamin D compared to the first-trimester ones. Furthermore, hypothyroidism and vitamin D deficiency have been reported to be correlated with preeclampsia.³¹ Therefore, upregulation of α 1-antitrypsin along with DBP in the third-trimester placentas could also be considered as a normal physiological response for assuring a successful pregnancy.

Figure 2. Analysis of the expression of protein disulfide isomerase and vitamin D binding protein using Western blot technique. A, After separation of total protein extracts on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), immune blotting was performed with appropriate antibodies. B, Quantitative analysis of the blotted band intensities. The bars represent the mean intensity $\pm$ standard deviation (SD) of the 6 first- and the 6 third-trimester placentas. * $P < .004$ for first- compared with third-trimester placenta, Mann-Whitney U test.

The last protein that showed downregulation in the late first-trimester placentas was EFHD1. The EFHD1 is an EF-hand domain-containing protein that displays increased expression during neuronal differentiation.³² Although no data have been published about the role of this protein in the human placenta, overexpression of EFHD1 in term placentas may result from fetal neuronal system's differentiation, a process which is more active in the third compared to the first trimester.³³ Recently, Morte et al indicated that thyroid hormones were the main regulator of the fetal brain development in rats.³⁴ This observation is in line with our findings regarding the upregulation of DBP and α -1-antitrypsin as well as overexpression of EFHD1 in full-term placentas.

Although the present study introduces a group of altered proteins in the first compared with the third trimester human placenta, repeating this study with a larger sample size is recommended. Moreover, due to ethical considerations normal first-trimester placentas were not available to this study.

Therefore, first-trimester placentas were collected from women who had permission for legal abortion. Although these cases did not receive additional medications and entered to the study after ensuring normal placental pathology, confirmation of our results using first-trimester placentas derived from normal pregnant women is highly recommended. On the other hand, since the same method for delivery of the placenta has been used in all cases, differential expression of the reported proteins could not be related to removal of the placenta.

In summary, the findings of the present study as the first research comparing the first- and third-trimester placental proteomes introduced a group of 11 proteins with altered expression. Interestingly, some of these proteins have been reported to be altered in pregnancy-related disorders.

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