RESEARCH ARTICLE

Proteomics study reveals that the dysregulation of focal adhesion and ribosome contribute to early pregnancy loss

Lingli Xin[1]**, Benhong Xu[2]**, Li Ma[1], Qingxiang Hou[1], Mei Ye[1], Shu Meng[2], Xiaoping Ding[1] and Wei Ge[2]*

[1] Department of Obstetrics and Gynecology, the General Hospital of the PLA Rocket Force, Beijing, China
[2] National Key Laboratory of Medical Molecular Biology, Department of Immunology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China

Purpose: Early pregnancy loss (EPL) affects 50–70% pregnant women in first trimester. The precise molecular mechanisms underlying EPL are far from being fully understood. Therefore, we aim to identify the molecular signaling pathways relating to EPL.
Experimental design: We performed proteomics and bioinformatics analysis of the placental villi in women who have undergone EPL and in normal pregnant women. The proteomics data were validated by Western blot analysis.
Results: We identified a total of 5952 proteins in placental villi, of which 588 proteins were differentially expressed in the EPL women. Bioinformatics analysis revealed that these differentially expressed proteins participated in a variety of signaling pathways, including the focal adhesion pathway and ribosome pathway. Moreover, results of the Western blot confirmed that Desmin, Lamin A/C, MMP-9, and histone H4 were upregulated in EPL and the Lamin C/Lamin A ratio decreased obviously in EPL. These proteins could be associated with the pathophysiology of EPL. The data have been deposited to the ProteomeXchange with identifier PXD002391.
Conclusion and clinical relevance: Our study demonstrated that the focal adhesion pathway and ribosome pathway are involved in EPL, and these findings might contribute to unveil the pathophysiology of EPL.

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1 Introduction

Early pregnancy loss (EPL) occurs before 12 wk of gestation, and is the most common complication of human reproduction [1, 2]. The etiologies of EPL include chromosomal defects, endocrine diseases, anatomical abnormalities of the female genital tract, infections, immunological factor, chemical agents, hereditary disorders, and psychological factors [1].

However, the molecular mechanisms of EPL are still poorly understood. Often, the cause of EPLs cannot be identified. Moreover, most of EPLs occur during the first month after the last menstrual period, and are often ignored as they occur during an expected menstrual cycle [3]. These preclude the prophylaxis and diagnosis of EPL to a great extent. Moreover, EPL has adverse effects on the quality of life and health of women. Therefore, it will be of great benefit to investigate the molecular mechanisms of the EPL.

The molecular mechanisms of embryonic development involve a variety of signaling pathways. Several lines of

Correspondence: Dr. Xiaoping Ding, Department of Obstetrics and Gynecology, the General Hospital of the PLA Rocket Force, Xinjiekou Outer Street 16#, Xicheng district, Beijing 100088, China
E-mail: dxp9902@tom.com

Abbreviations: ECM, extracellular matrix; **EPL**, early pregnancy loss; **TBST**, TBS with Tween 20; **TMT**, tandem mass tag

*Additional corresponding author: Dr. Wei Ge
E-mail: wei.ge@chem.ox.ac.uk
**These authors contributed equally to this work.
Colour Online: See the article online to view Figs. 1–3 in colour.

Clinical Relevance

In the present study, we applied TMT-labeled pro-teomics to demonstrate that the mechanisms of early pregnancy loss (EPL) involve a number of pathways, including the focal adhesion pathway and ribosome pathway, and Desmin, the Lamin C/Lamin A ratio, histone H4, and MMP-9 are critical for EPL and their expression alternation might contribute to the patho-physiology of EPL. This study proved that proteomics may be a useful tool for identifying underlying molec-ular mechanisms for EPL. Moreover, it might provide novel targets for treatment of EPL and reduce the incidence of EPL.

evidence have demonstrated that abnormal metabolism of ROS would lead to miscarriage [4]. Sharp et al. indicated that a delicate balance between apoptosis and macroautophagy is critical to normal development of the villous tree [5]. Increased expression of genes related to apoptosis inhibition, including ARRb1 and FYN, have been observed in placental villi dur-ing the first trimester [6]. Previous studies have also demon-strated that the involvement of the mTOR/FRAP-1 pathway in the developmental process of placentation and embryoge-nesis, and mTOR/Frap1 null mice were shown to have failed pregnancies with inhibited embryogenesis and trophoblast growth [7, 8]. Furthermore, previous studies found that the calcium-dependent cell–cell adhesion protein E-cadherin de-creases in chorionic villi of EPL [9, 10]. In addition, Callejia-Agius et al. suggested that the inflammatory pathways also participated in EPL and that increased TNF-α and IL-10 ex-pression in villous tissue played an essential role in EPL [11]. Khan et al. demonstrated using genome wide analysis that ap-proximately 9000 genes with known functions show marked expression level while displaying a defined spatiotemporal profile in normal placental villi during first trimester [6].

However, current investigating tools can only test one or a few specific proteins at a time. Therefore, compara-tive analysis of global protein expression would be helpful for exploring the molecular signaling pathways involved in EPL. Proteomics facilitates large-scale studies of proteins and enables a comprehensive analysis of the functions and sig-naling pathways in which the proteins participate. Liu et al. performed 2D gel-based proteomics to explore differentially expressed proteins between EPL and normal placental vil-lous tissues. They found that 13 proteins, including Fas inhibitory molecule (FAIM), S100 calcium-binding protein A11 (S100A11), and RNA-binding protein regulatory sub-unit, were downregulated in EPL, and five proteins, includ-ing ubiquitin-conjugating enzyme E2N (UBE2N) and pro-teasome beta-subunit, were significantly upregulated in EPL [12]. However, only the most abundant proteins can be ob-served using this method [13]. Ni et al. conducted a gel-feel tandem mass tag (TMT) labeling based proteomics analysis to compare the differentially expressed proteins in placen-tal villous tissues from normal pregnant women and EPL patients, and the proteins that showed altered expression were different from those reported in the previous study. They found 51 differentially expressed proteins, of which 22 proteins were upregulated and 29 proteins were downregulated, including glutathione S-transferase mu 2 (GSTM2), BC1 (ubiquinol-cytochrome c reductase) synthesis-like (BCS1L), and cullin 7 (CUL7). Moreover, they used path-way analysis and demonstrated that these differentially ex-pressed proteins participated in cell migration, angiogenesis, oxidative stress, apoptosis, and metabolism pathways [14]. Given the inconsistences between the abovementioned two studies, the molecular signaling pathways associated with EPL require further investigation.

To comprehensively study the molecular signaling path-ways related to EPL, we used TMT labeling-based proteomics analysis to identify differentially expressed proteins between placental villi from EPL and normal pregnant women. Then, we performed bioinformatics analysis to identify the molec-ular pathways involved in EPL. Moreover, we used Western blot analysis to confirm the proteomics data. Inconsistent to previous proteomics study, we suggested that EPL involved a variety of signaling pathways, including the focal adhesion pathway and ribosome pathway, and that Desmin, Lamin A/C, MMP-9 play critical roles in EPL. Furthermore, this is the first study to show that increased expression level of his-tone H4 plays a role in EPL.

2 Materials and methods

2.1 Sample collection and preparation

The placental villi of EPL patients and normal pregnant women obtained between October 2014 and December 2014 were included in the present analysis. Informed consent was obtained from all the subjects. This study was approved by the Ethical Review Committee of The Second Artillery Gen-eral Hospital of Chinese PLA (Beijing, China). Gestational sacs without fetal heart rate identified by transvaginal ultra-sound were diagnosed as EPL. Age-matched women with normal pregnancies undergoing intentional terminations of pregnancy at the same gestation age were included as con-trol group. The following inclusion criteria were applied as described previously [12]: women who underwent termina-tion at a gestational age at 5–10 wk (based on the first day of the last menstrual period) and had no history of re-current spontaneous abortions, chromosomal abnormalities,

endocrine diseases, anatomical abnormalities of genital tract, infections, immunologic diseases, trauma, internal diseases, or any chemical agent intake before their terminations. Placental villi were obtained very carefully through the cervix during dilatation and aspiration according to strict clinical procedures. Then, the samples were washed thoroughly with ice-cold PBS, and deciduas were removed carefully. Thereafter, the karyotype assays were performed to exclude villi with chromosomal abnormalities. Finally, five EPL villi and five normal villi were selected. The placental villi were maintained in −80°C until further proteomics analysis.

2.2 Protein extraction and TMT labeling

The placental villi of both groups were immediately submerged in liquid nitrogen after being weighted, and the tissues were then ground to powder. Then, the placental villi were lysed in lysis buffer (8 M urea (GE Healthcare, 17-1319-01) in PBS (pH 8.0), 1× proteinase inhibitor (Roche), 1 mM PMSF), and the lysates were centrifuged at 12 000 × *g* at 4°C for 15 min. The supernatants were collected and the protein concentrations were measured by Nanodrop 2000 at an absorbance of 280 nm. Then, the supernatants of both groups were mixed, respectively; 100 μg of protein of each group was incubated with 10 mM dithiothreitol at 55°C for 1 h, and alkylated with 25 mM indole acetic acid at room temperature in the dark. The proteins were then digested with trypsin/Lys-C Mix (Promega, V5072) at 37°C overnight (protease:protein ratio of 1:25). Thereafter, protein digests extracted from EPL group and control group were labeled with 0.8 mg TMT[6]-131 or TMT[6]-130 (Thermo Scientific, 90061), respectively. Equal amounts (100 μg) of labeled protein digests from both groups were mixed for MS.

2.3 HPLC

The combined TMT-labeled protein digests were fractionated with HPLC analysis (UltiMate 3000 UHPLC, Thermo Scientific) using an Xbridge BEH300 C18 column (4.6 × 250 mm^2, 5 μm, 300 Å; Waters). Fifty fractions were collected into microtubes at 1.5 min intervals. All the fractions were dried in a vacuum concentrator and dissolved in 20 μL of 0.1% formic acid for further LC–MS/MS analysis.

2.4 LC–MS/MS analysis

A Q Exactive mass spectrometer was used for the LC–MS/MS analysis. The protein digests were separated using a 120 min elution gradient at a flow rate of 0.3 μL/min in an UltiMate 3000 RSLCano System (Thermo Scientific), and analyzed with a directly interfaced Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific). A homemade fused-silica capillary column (75 μm × 150 mm, Upchurch, Oak Harbor, WA, USA) packed with C18 resin

(300 Å, 5 μm, Varian Lexington, MA, USA) was used as the analytical column. Xcalibur 2.1.2 software was used with the Q Exactive mass spectrometer in data-dependent acquisition mode. After ten data-dependent MS/MS scans were obtained at 27% normalized collision energy, a single full-scan mass spectrum in Orbitrap (400–1800 *m/z*, 60 000 resolutions) was performed.

2.5 Western blot analysis

Western blot analysis of proteins from four EPL, four normal placental villi, and pooled samples of both groups was performed according to standard procedure with minor modifications. Equal amounts of total proteins of each subject (20 μg) were subjected to 12% SDS-PAGE and transferred to NC membranes. Membranes blockage was performed at room temperature for 1 h in TBS with Tween 20 (TBST) with 5% nonfat milk. The membranes were then incubated with anti-Desmin (ab32362), anti-Lamin A/C (ab108922), anti-histone H4 (ab10158), anti-MMP-9 (ab137867), and anti-β-beta actin (internal control) antibody (GTX124213) at 4°C overnight. The membranes were washed in TBST for 15 min and incubated with goat anti-rabbit HRP-conjugated IgG for 1 h at room temperature. The membranes were washed three times in TBST, and chemiluminescence was performed with ECL detection kits according to the manufacturer's instructions.

2.6 Data analysis

LC–MS/MS data were searched against the human FASTA database from UniProt (released on Dec 9, 2015) using the Thermo Scientific Proteome Discoverer software suite 1.4 with the SEQUEST search engine. The parameter settings were as follows: full trypsin specificity, two missed cleavages, carbamidomethylation (C), and TMT 6-plex (K and peptide N-terminal) as the static modification, oxidation (M) as the dynamic modification, precursor ion mass tolerances were set at 20 ppm for all MS data acquired using an Orbitrap mass analyzer, and the fragment ion mass tolerance was set as 20 mmu for all MS/MS spectra acquired. At least one unique peptide per protein had to be identified to list the protein as a hit. Fold changes were calculated by the ratio of proteins labeled with TMT6-131 and TMT6-130 adjusted by the β-actin ratio value. The thresholds for downregulation and upregulation were set at 0.8 and 1.2, respectively. The proteins that scored equal or greater than 10 were selected for bioinformatics analysis. Then, the UniprotKB/Swiss-Prot accession numbers were converted into Entrez Gene IDs for subsequent analysis by WebGestalt online toolkit (www.bioinfo.vanderbilt.edu/webgestalt/). The FunRich software 2.1.2 (http://www.funrich.org) was used to classify the proteins, and the significance level was set at 0.001. The WebGestalt online toolkit was also used to

Table 1. Comparison of subject characteristics between the EPL group and normal group

Subject characteristics	Group	Mean ± SD (*n* = 5)	*p*-Value
Age (years)	EPL	30.6 ± 2.015	0.54
	Control	29.0 ± 1.517	
Pregnancy duration (days)	EPL	47.0 ± 6.301	0.57
	Control	42.8 ± 3.338	
Number of previous pregnancies	EPL	1.8 ± 0.374	0.69
	Control	2.0 ± 0.316	

conduct enrichment analysis based on Wikipathways and KEGG analysis databases. The significance level was set at 0.0001. The differentially expressed proteins in matched pathways were visualized using Cytoscape software (version 3.1.1). The MS proteomics data have been deposited to the ProteomeXchange Consortium [15] via PRIDE partner repository with the data identifier PXD002391.

3 Results

3.1 Subjects characteristics

The subjects showed no significant differences in age, pregnancy duration, and number of previous pregnancies. Thus, the subjects included in the present study showed relatively good consistency. Consequently, it can be said that in this study the EPL was not correlated with age, pregnancy duration, or number of previous pregnancies. Detailed information is provided in Table 1.

3.2 Differentially expressed proteins

To compare the differentially expressed proteins between EPL and normal placental villi, we analyzed the proteome of the placental villi from both groups using proteomics analysis. A total of 5952 proteins were identified, and four were excluded as no ratio values between the two groups were obtained for these proteins. Of the remaining 5948 proteins, 3109 proteins scored over 10 were extracted for subsequent analysis. Thereafter, we compared the expression level of the remaining 3109 proteins between EPL group and control group. Of these, 588 proteins were differentially expressed proteins: 135 proteins were upregulated and 453 proteins were downregulated in the EPL group relative to the control group. (Supporting Information File 1).

3.3 Classification of the differentially expressed proteins

To classify the differentially expressed proteins, enrichment analysis was performed using FunRich software 2.1.2. The

UniProt IDs of these differentially expressed proteins were automatically converted into Enrtez Gene IDs by FunRich. The 588 differentially expressed proteins were encoded by 592 genes, 13 of which were not mapped with the database. These mapped proteins were classified according to cellular component, biological process, and molecular function. In the context of cellular component, 547 genes were matched, and the proteins encoded were mainly components of exosomes (41%), lysosome (27.6%), centrosome (15%), extracellular matrix (ECM, 6%), cytoplasm (57.6%), and extracellular space (10.2%) (Fig. 1A and Supporting Information File 2). According to the biological process, 573 out of 592 genes matched the database. Our results showed that these differentially expressed proteins were mainly significantly involved in the biological process of energy pathways (16.6%), metabolism (16.9%), cell growth and/or maintenance (11.7%), and protein metabolism (13.1%) (Fig. 1B and Supporting Information File 2). In the context of molecular function, the differentially expressed proteins mainly involved in ECM structural constituent (4.2%), RNA binding (5.6%), catalytic activity (6.5%), isomerase activity (1.4%), structural constituent of ribosome (2.6%), and complement activity (1%) were enriched (Fig. 1C and Supporting Information File 2). Taken together, our results showed that the proteins with a variety of molecular functions and biological processes contributed to the pathophysiology of EPL.

3.4 Pathways involving the differentially expressed proteins

The WebGestalt online toolkit was used to perform enrichment analysis based on the Wikipathways and KEGG pathway databases in order to identify the signaling pathways in which the differentially expressed proteins were involved. Fourteen signaling pathways with high confidence were identified based on Wikipathways database (Table 2). Of these 14 signaling pathways, the focal adhesion pathway and the cytoplasmic ribosomal proteins pathway involved 16 and nine differentially expressed proteins, respectively. Enrichment analysis based on KEGG pathway database demonstrated that the differentially expressed proteins were involved in 25 pathways, including the focal adhesion pathway and the ribosome pathway (Supporting Information File 3). Based on KEGG pathway database, the metabolic pathway was found to involve the maximum number of proteins (66 proteins). Taken together, our results suggested that interferences in the focal adhesion pathway and cytoplasmic ribosomal protein pathway contribute to EPL. Cytoscape software was used to visualize the differentially expressed proteins in these two pathways. In the focal adhesion pathway, nine of ten proteins participating in ECM–receptor interactions were obviously upregulated, including COL4A1, FN1, LAMA2, LAMA5, LAMB1, TNC, VTN, LAMC1, and TNXB, while only LAMA1 was downregulated. Nevertheless, the intracellular AKT2 and PAK2 decreased slightly (Fig. 2). In the cytoplasmic ribosomal protein
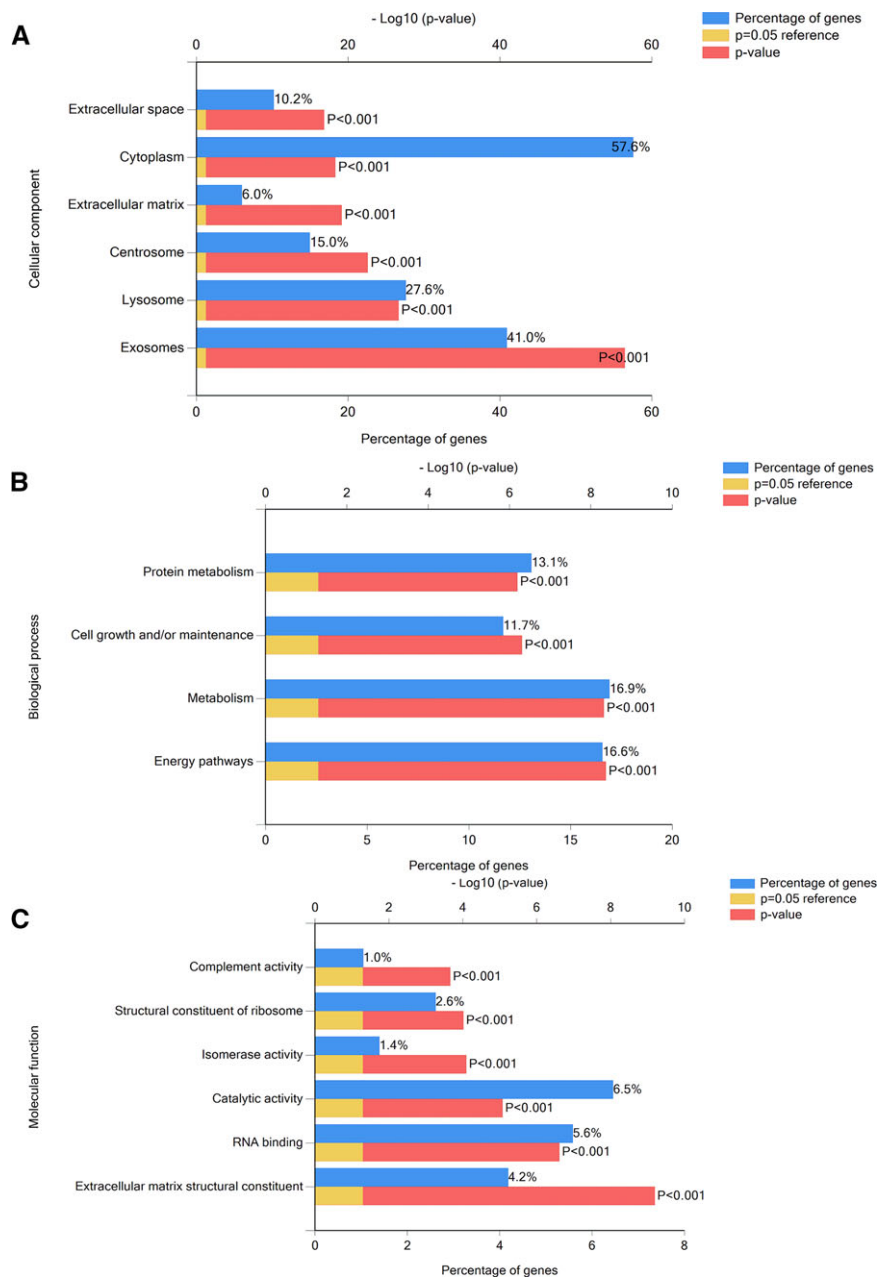
Figure 1. Enrichment analysis of the differentially expressed proteins by FunRich. (**A**) Bar chart of the enriched cellular component that differentially expressed proteins implicated in the database. (**B**) Bar chart of the enriched biological process that differentially expressed proteins implicated in the database. (**C**) Bar chart of the enriched molecular function that differentially expressed proteins implicated in the database.

pathway, almost all proteins matching Wikipathways database were downregulated, suggesting that functions of ribosomes were obviously inhibited in EPL (Fig. 3).

3.5 Western blot analysis of the differentially expressed proteins

To validate the results of the proteomics data, we performed Western blot analysis of four relevant proteins in four EPL samples, four control samples, and pooled samples from both groups based on proteomics and bioinformatics analyses. The four selected proteins were Desmin, Lamin A/C,

MMP-9, and histone H4, and their fold changes according to the proteomics analysis were 2.13, 1.33, 1.22, 1.30, and 1.30, respectively. Western blot analysis (Fig. 4) confirmed that histone H4 and Desmin were markedly upregulated in EPL group compared with the control group. Western blot analysis showed that MMP-9 was significantly upregulated in the EPL group, though proteomics data showed marginal increase in the EPL group. The expression of Lamin C was approximately equal to that of Lamin A in EPL. In contrast, the expression of Lamin C was obviously higher than that of Lamin A in the control group. The Lamin C/Lamin A ratio was obviously decreased in the EPL group relative to the control. Collectively, our findings confirmed the results of

Table 2. Pathways in which differentially expressed proteins are involved, based on Wikipathway database

Pathway name	Gene no.	Entrez Gene	Statistics
Glycolysis and gluconeogenesis	13	6515 2597 2806 5230 3939 4190 2805 2821 5315 2023 5223 7167 3945	$C = 50$; $O = 13$; $E = 0.66$; $R = 19.71$; raw$p = 7.28 \times 10^{-14}$; adj$p = 7.50 \times 10^{-12}$
Epithelium TarBase	23	10105 5236 5270 3915 3035 4677 5725 10061 10971 4691 51809 5315 4144 10797 488 83858 23603 822 396 5878 1021 143888 4907	$C = 340$; $O = 23$; $E = 4.49$; $R = 5.13$ raw$p = 2.37 \times 10^{-10}$; adj$p = 1.22 \times 10^{-8}$
Lymphocyte TarBase	28	10105 10484 5236 5270 3915 3035 4677 5725 26973 10061 10971 4691 51809 5315 4144 11137 10797 488 83858 23603 822 396 6902 10642 5878 1021 143888 4907	$C = 533$; $O = 28$; $E = 7.03$; $R = 3.98$ raw$p = 8.98 \times 10^{-10}$; adj$p = 3.08 \times 10^{-8}$
Focal adhesion	16	2335 3915 208 3684 7148 3908 284217 3371 3689 7448 2321 3912 5062 1282 3694 3911	$C = 185$; $O = 16$; $E = 2.44$; $R = 6.56$ raw$p = 4.07 \times 10^{-9}$; adj$p = 1.05 \times 10^{-7}$
Statin pathway	7	6646 341 336 348 6713 5360 335	$C = 30$; $O = 7$; $E = 0.40$; $R = 17.69$ raw$p = 1.05 \times 10^{-7}$; adj$p = 2.16 \times 10^{-6}$
Leukocyte TarBase	13	10105 5270 26973 10971 5315 10797 488 23603 822 396 6902 5878 1021	$C = 160$; $O = 13$; $E = 2.11$; $R = 6.16$ raw$p = 2.40 \times 10^{-7}$; adj$p = 3.61 \times 10^{-6}$
Vitamin A and carotenoid metabolism	8	1382 948 5950 54884 8854 220 216 5947	$C = 49$; $O = 8$; $E = 0.65$; $R = 12.37$; raw$p = 2.45 \times 10^{-7}$; adj$p = 3.61 \times 10^{-6}$
Muscle cell TarBase	21	10105 10484 5236 3915 26973 10971 4691 51809 5315 11137 10797 488 83858 23603 822 396 6902 10642 1021 143888 4907	$C = 424$; $O = 21$; $E = 5.59$; $R = 3.75$ raw$p = 3.03 \times 10^{-7}$; adj$p = 3.90 \times 10^{-6}$
mRNA processing	11	3178 3182 5725 3183 22826 10236 27316 3184 3191 4904 3192	$C = 132$; $O = 11$; $E = 1.74$; $R = 6.32$ raw$p = 1.57 \times 10^{-6}$; adj$p = 1.80 \times 10^{-5}$
Cytoplasmic ribosomal proteins	9	6202 6154 6230 6204 4736 3921 6147 6201 6223	$C = 88$; $O = 9$; $E = 1.16$; $R = 7.75$ raw$p = 2.58 \times 10^{-6}$; adj$p = 2.66 \times 10^{-5}$
Complement and coagulation cascades	7	718 2 336 5265 5340 2244 717	$C = 51$; $O = 7$; $E = 0.67$; $R = 10.40$ raw$p = 4.70 \times 10^{-6}$; adj$p = 4.03 \times 10^{-5}$
Translation factors	7	1984 1975 7458 5610 1938 10209 26986	$C = 51$; $O = 7$; $E = 0.67$; $R = 10.40$ raw$p = 4.70 \times 10^{-6}$; adj$p = 4.03 \times 10^{-5}$
AGE-RAGE pathway	8	4318 6647 3958 5578 1729 5579 6772 4478	$C = 76$; $O = 8$; $E = 1.00$; $R = 7.98$ raw$p = 7.50 \times 10^{-6}$; adj$p = 5.94 \times 10^{-5}$
G13 signaling pathway	6	397 1729 10788 5216 9138 1072	$C = 37$; $O = 6$; $E = 0.49$; $R = 12.29$ raw$p = 8.44 \times 10^{-6}$; adj$p = 6.21 \times 10^{-5}$

C, the number of reference genes in the category; *O*, the number of genes in the gene set and also in the category; *E*, the expected number in the category; *R*, ratio of enrichment; raw*p*, p-value from hypergeometric test; adj*p*, p-value adjusted by the multiple test adjustment.

proteomic analysis that Desmin, Histone H4 and MMP-9 up-regulated and the Lamin C/Lamin A ratio was downregulated in the EPL group compared to the control group and all these proteins may serve as biomarkers for EPL.

4   Discussion

In the present study, we used proteomics analysis to identify a total of 5952 proteins expressed in placental villi. Of these, 588 were differentially expressed between the EPL and normal groups. Subsequent bioinformatics analysis demonstrated that these differentially expressed proteins implicated in a number of biological processes and molecular functions. Moreover, our results revealed that a variety of molecular pathways, including the focal adhesion pathway and ribosome pathway, were disrupted in the EPL but not in the normal pregnancies. Given that histones and Lamin A/C play a role in gene regulation, while Desmin and MMPs are critical in focal adhesion pathway, we focused on four proteins:

Desmin, Lamin A/C, MMP-9, and histone H4, and speculated that these proteins could be associated with EPL.

Focal adhesion is a critical signaling pathway at the interface between cells and the ECM. The ECM interacts with membrane-associated multicomponent complexes to transduce mechanosensory signaling and controls multiple cell functions [16, 17]. Kaneko et al. showed that focal adhesion and the focal adhesion proteins are essential for successful implantation and placentation during early pregnancy. They reported redistribution of two focal adhesion proteins, talin and paxillin, during early pregnancy in rats [18]. Our results revealed that 16 differentially expressed proteins participated in the focal adhesion pathway, of which ten were involved in ECM–receptor interactions. Moreover, nine of the ten proteins (COL4A1, FN1, LAMA2, LAMA5, LAMB1, TNC, VTN, LAMC1, and TNXB) involved in ECM–receptor interactions were upregulated while intracellular AKT2 and PAK2 were downregulated (Fig. 2), indicating that the intracellular focal adhesion turnover signaling was slightly inhibited. In addition, our results also showed the upregulation of ITGAM in integrin alpha complex and ITGB2, ITGB6 in integrin beta
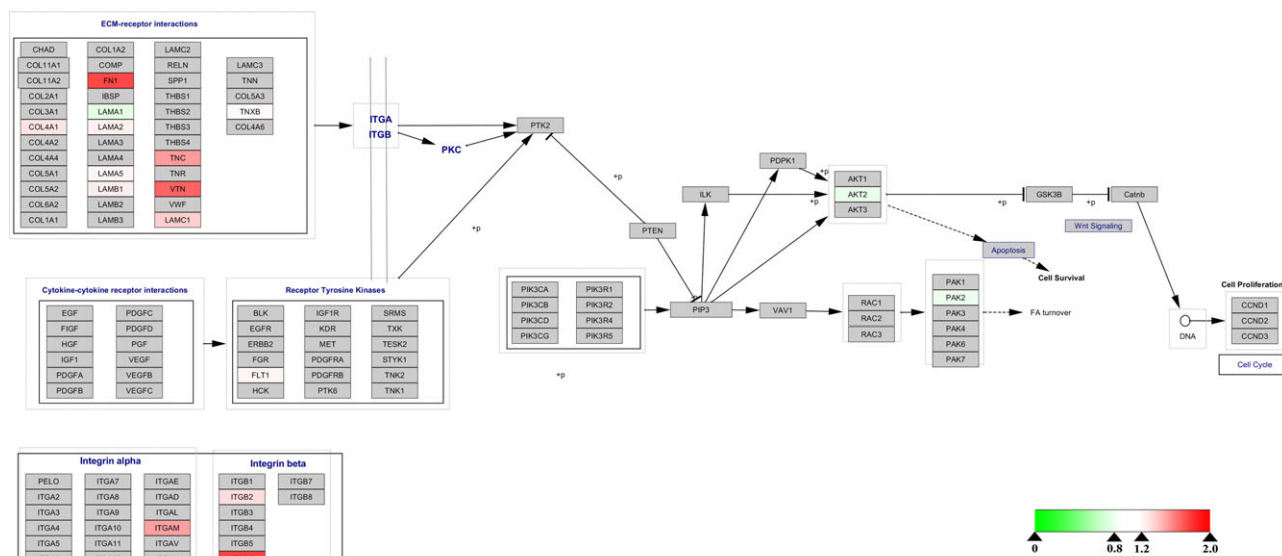
Figure 2. Visualization of the differentially expressed proteins in the focal adhesion pathway by Cytoscape software. Red boxes indicate upregulated proteins; green boxes indicate downregulated proteins; gray boxes indicate proteins unidentified in present study; and white boxes indicate proteins with unchanged expression level between the EPL group and control group. The values of fold changes are demonstrated by color intensity.

complex. Similar to our results, Shaw et al. also reported the involvement of LAMA5 and LAMB1 in early pregnancy [19]. Upregulation of proteins related to focal adhesion is involved in the increased myometrial contractility, which always occurs at the end of pregnancy [20, 21]. In our study, we found that the increased expression of proteins is associated with focal adhesion in early pregnancy in EPL patients. These findings implied that overexpression of proteins associated with focal adhesion pathway at the early stage of pregnancy played a critical role in EPL. However, Tachibana et al. demonstrated that rather than attachment through focal adhesion, conceptus chorionic membrane ECMs only function as a scaffold-like structure [22], suggesting further studies are needed to address the inconsistence.

In addition, our results indicated that functions of ribosomes were inhibited in EPL. Ribosomes played important roles in translating mRNAs and building proteins, which are essential for metabolic processes [23]. Our proteomics data showed that 97 proteins of metabolic process were changed (Supporting Information File 2). Thus, our results implied that inhibited ribosomal functions disrupted the metabolic processes, which eventually contributed to EPL.

Lamin A/C are two major isoforms of A-type lamins that play an important role in many nuclear fundamental processes, including DNA replication and repair and RNA transcription and splicing [24]. Ko et al. found that apoptosis-related Lamin A was significantly upregulated in the somatic cell nuclear transfer-derived conceptuses compared with conceptuses derived from natural matings [25]. Foster et al. reported that in normal porcine embryo development, Lamin A is found in one-cell, two-cell, four-cell, and six- to eight-

cell stages, and it reduces in later embryonic stages [26]. In the present study, proteomics analysis showed that the levels of Lamin A/C increased in EPL relative to the control. Furthermore, results of the Western blot analysis indicated that Lamin A was significantly upregulated in EPL while lamin C showed a slightly increase. The Lamin C/Lamin A ratio decreased obviously in EPL relative to the control. Taken together, we speculated that increased Lamin A content at least partly contributes to EPL, and the turnover ratio of Lamin C/Lamin A might be a valuable diagnostic parameter in EPL.
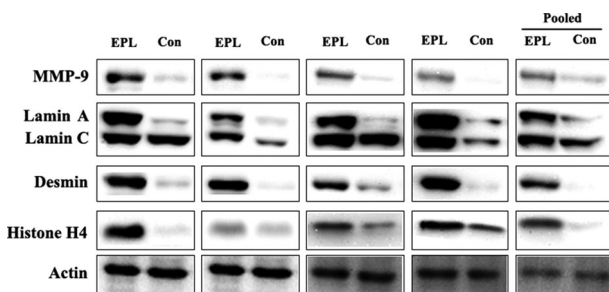
In eukaryotic organisms, the DNA is packaged in chromatin, whose predominant components are histone H2A, H2B, H3, and H4 [27, 28]. Histones modifications, including acetylation, methylation, and phosphorylation, can modulate DNA transcription via activating or repressive signals [29]. Previous studies have showed that histone modifications play a role in embryonic development [30, 31]. Tachibana et al. reported that H3K9 methylation is essential for early embryogenesis, and that deletion of histone methyltransferase G9a reduced H3K9 methylation and suppressed transcription of developmental genes [32]. To the best of our knowledge, ours is the first study to demonstrate that histone H4 protein levels were upregulated in EPL. However, the molecular insights remain elusive. Therefore, we implied that upregulated histone H4 might lead to EPL through unidentified mechanisms other than histone modifications.

Desmin is a muscle-specific protein present early in the development of muscle cells and is associated with several functions. However, it is present in low levels during embryogenesis, and the Desmin content in cells increases
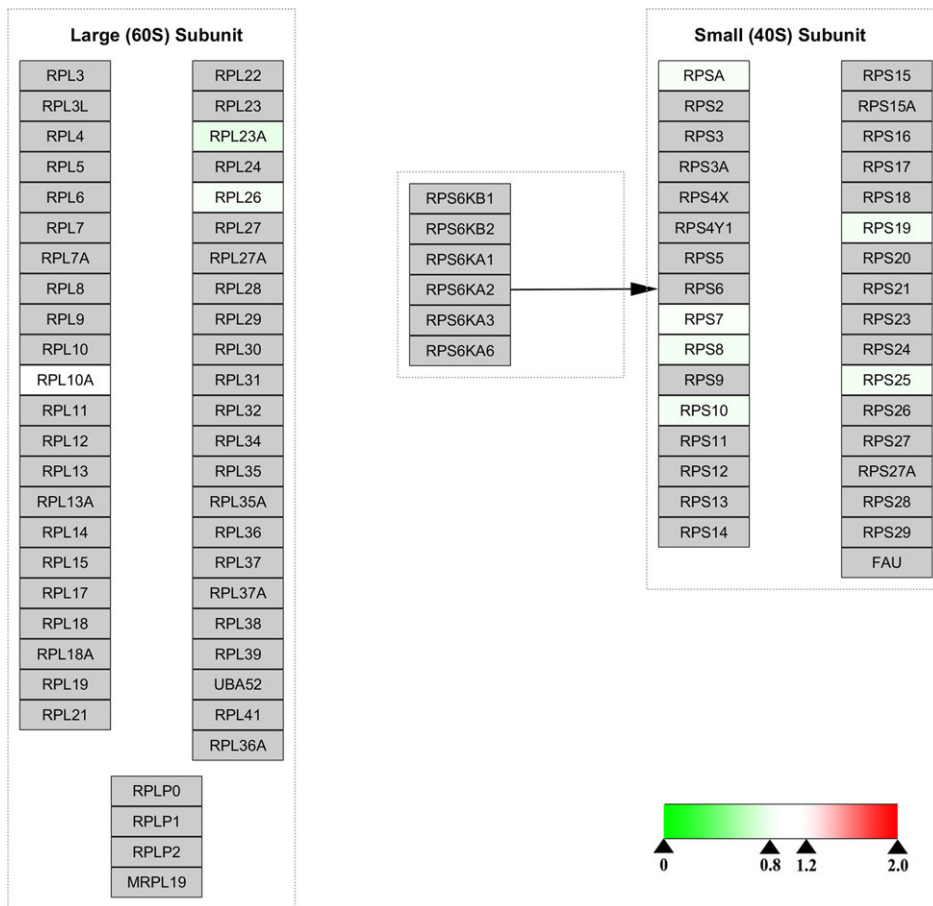
Figure 3. Visualization of the differentially expressed proteins in the cytoplasmic ribosomal proteins pathway by Cytoscape software. Red boxes indicate upregulated proteins; green boxes indicate downregulated proteins; gray boxes indicate proteins unidentified in present study; white boxes indicate proteins whose expression level was unchanged between EPL group and control group. The values of fold changes are demonstrated by color intensity.

as the cells near terminal differentiation [33]. Kishi et al. found that strong Desmin expression was observed after E13 and continued until maturity in mylohyoid muscle [34]. Previous studies have suggested that Desmin is a sensitive biomarker for endothelial cell differentiation, embryonal sarcomas, and colorectal cancer [35, 36]. As cytotrophoblast cells in early pregnancy share common characteristics with ma-

lignant cells, and our results showed involvement of 17 proteins in pathways in cancer (Supporting Information File 3), it is reasonable to speculate that Desmin participates in pregnancy. In fact, Sak et al. found that the Desmin expression in placental villi was higher in patients with HELLP syndrome than in normal pregnant women. They suggested that elevated Desmin inhibited endothelial cell movements and affected normal placental functions [37]. Our results revealed that the Desmin levels were markedly increased in the villi of EPL women. Taken together, our study indicated that Desmin might be related to the pathology of EPL.

MMP-9 is an inducible enzyme of the zinc metalloproteinases family related to the degradation of the ECM, which is shown to relate to invasion of cytotrophoblast cells into the endometrium and the myometrium, and are involved in spiral artery formation during implantation [38]. Whiteside et al. found that ECM-degrading activity of mouse embryos was prohibited by MMP-9 antisense oligonucleotides [39]. However, Singh et al. found that a single-nucleotide polymorphism in the MMP-9 promoter was not correlate with recurrent pregnancy loss [40]. Moreover, Anumba et al. indicated that the serum levels of MMP-9 did not differ between women with and without a history of recurrent pregnancy loss [41]. Inconsistent with previous studies, our results showed

Figure 4. Western blot analysis of MMP-9, Lamin A/C, Desmin, histone H4 between the EPL and normal placental villi. MMP-9, histone H4, and Desmin were markedly upregulated in EPL relative to the control. The ratio of Lamin C/Lamin A was obviously decreased in the EPL compared to the control. EPL, early pregnancy loss; Con: control.

that MMP-9 was upregulated in women with EPL compared to normal pregnant women. Jiang et al. reported that the levels of MMP-9 mRNA was significantly higher in spontaneous abortion than the corresponding levels in normal pregnant women. Moreover, they suggested that TIMP-3 mRNA expression levels were similar in cases of spontaneous abortion and normal abortion [42]. However, our proteomic data showed that the TIMP-3 levels was upregulated 1.42-fold in EPL (Supporting Information File 1). These might suggest that the mechanisms underlying EPL and spontaneous abortion are different. In addition, because TIMP-3 is the major inhibitor of MMP-9, increased TIMP-3 is probable a feedback mechanism for upregulated MMP-9 in EPL. Taken together, these findings imply that upregulation of MMP-9 contributes to EPL by disrupting invasion of the embryo into the maternal endometrium.

Notably, our study identified a few different differentially expressed proteins from previous proteomics studies by Liu et al. and Ni et al. [12, 14], although the functions of most proteins are in common between our study and each of the other. The differences might be because we used TMT labeling based proteomics analysis, while Liu et al. used 2D gel-based proteomics analysis. In addition, the subjects included in our study were mainly from northern China, while those included in Ni et al. study were from southern China. Moreover, the gestational age in our study was 5–10 wk, which was different from 7 to 8 wk in study by Ni et al. These might partially contribute to the different differentially expressed proteins in our results compared with published papers.

In addition, there are some limitations in our study. First, there were only five EPL villi and five control villi samples included, and the demographic information of the subjects is limited. Considering the little number of the samples and little demographic information, the results in this study should be interpreted cautiously and further studies including large number of samples and more specific demographic information are required. Second, due to the technical limitations, the maternal decidua could not be excluded completed. The transcriptome analysis of decidua demonstrated the expression of genes related to coagulation system, macrophage stimulation, calcium signaling, nucleotide binding, microsome, and vesicular fraction were changed [43,44], which are different from our results. It possibly suggested that the altered proteins in our study mainly origin from placental villi. Advanced sample collecting technique should be included in the studies of EPL to exclude the interference of the maternal factors.

In conclusion, our study suggested that the mechanisms of EPL involve a number of pathways, including the focal adhesion pathway and ribosome pathway, and Desmin, the Lamin C/Lamin A ratio, histone H4 and MMP-9 are critical for EPL and their expression alternation might contribute to the pathophysiology of EPL. Our study also proved that proteomics is a useful tool for identifying molecular mechanisms for EPL.

5　References

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