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Proteomics and bioinformatics analysis of altered protein expression in the placental villous tissue from early recurrent miscarriage patients

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ABSTRACT

Introduction

Recurrent miscarriage (RM) affects 5% of women, it has an adverse emotional impact on women. Because of the complexities of early development, the mechanism of recurrent miscarriage is still unclear. We hypothesized that abnormal placenta leads to early recurrent miscarriage (ERM). The aim of this study was to identify ERM associated factors in human placenta villous tissue using proteomics. Investigation of these differences in protein expression in parallel profiling is essential to understand the comprehensive pathophysiological mechanism underlying recurrent miscarriage (RM).

Methods

To gain more insight into mechanisms of recurrent miscarriage (RM), a comparative proteome profile of the human placenta villous tissue in normal and RM pregnancies was analyzed using iTRAQ technology and bioinformatics analysis used by Ingenuity Pathway Analysis (IPA) software.

Results

In this study, we employed an iTRAQ based proteomics analysis of four placental villous tissues from patients with early recurrent miscarriage (ERM) and four from normal pregnant women. Finally, we identified 2805 proteins and 79,998 peptides between patients with RM and normal matched group. Further analysis identified 314 differentially expressed proteins in placental villous tissue (≥1.3-fold, Student's *t*-test, $p < 0.05$); 209 proteins showed the increased expression while 105 proteins showed decreased expression. These 314 proteins were analyzed by Ingenuity Pathway Analysis (IPA) and were found to play important roles in the growth of embryo. Furthermore, network analysis show that Angiotensinogen (AGT), MAPK14 and Prothrombin (F2) are core factors in early embryonic development. We used another 8 independent samples (4 cases and 4 controls) to cross validation of the proteomic data.

Discussion

This study has identified several proteins that are associated with early development, these results may supply new insight into mechanisms behind recurrent miscarriage.

Abbreviations

FDR	false discovery rate
IPA	Ingenuity Pathway Analysis
SCX	strong cationic-exchange chromatography
DEPs	differentially expressed proteins
RM	recurrent miscarriage
ERM	early recurrent miscarriage

1. Introduction

Recurrent miscarriage (RM), the loss of two or more consecutive pregnancies prior to the 20th week of gestation; it is one of the most common clinical problems in reproduction, affects 5% of couples trying to conceive [1,2]. The known causes of RM include genetic or chromosomal abnormalities, endocrinological disorders, anatomic anomalies, and other such factors [3–5]. However, these related molecular mechanisms of recurrent miscarriage are still unexplained.

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The placenta is the organ that transports nutrients, respiratory gases, and wastes between the maternal and fetal systems [6]. In the early embryonic development, the placental barrier facilitates the embryonic growth and effectively avoiding radical damage; once embryogenesis is complete, the maternal intervillous circulation becomes fully established [7]. There is speculation that an abnormal placenta leads to early recurrent miscarriage (ERM), but the unclear relationship between the placenta and ERM remains requiring further elucidation.

In contrast to conventional biochemical approaches that monitor one or only a few specific proteins at a time, proteomics, particularly the combination of iTRAQ and 2D-LC-MS/MS, is a useful method to recognize altered protein expression as well as proteins involved in disease pathogenesis. In 2006, Kim, Y.S. et al. have identified for the first time recurrent miscarriage (RM)-associated proteins in follicular fluid of RM patients using 2D-gel-based proteomic tools [8]. They think that coagulation factors (fibrinogen γ and antithrombin) play an important role in maintaining the normal pregnancy. Later in 2011, they analyzed blood samples from normal and RM patients to conduct a comparative proteomic study, these results suggest that ITI-H4 expression may be used as a biomarker [9]. In 2014, Metwally, M. performed a proteomic analysis of the endometrium in obese and overweight women with recurrent miscarriage. These findings provide preliminary evidence for an alteration in the endometrial protein profile in overweight/obese women with recurrent miscarriage mainly in the form of increased haptoglobin, an inflammatory marker associated with obesity [10].

Nevertheless, up to date, there have been no reported attempts to screen the proteins associated with RM involved in placenta villous tissue, using large-scale proteomic analysis. Therefore, in this study, we aim to establish a comparative proteome profile of the human placenta villous tissue in normal and RM pregnancies using the iTRAQ technology coupled with 2D nano LC-MS/MS. In addition, we used Ingenuity Pathway Analysis (IPA) software to analysis these differentially expressed proteins to determined molecular function, downstream effects, and upstream regulator, which are useful for providing molecular insights into early recurrent miscarriage (ERM).

2. Methods

2.1. Clinical specimen collection and preparation

For this study, a total number of eight placental villous samples, four from ERM patients and four from elective abortions, were collected from Shaoxing women and children hospital in Zhejiang, China. Placental villous tissues were taken through the cervix during dilatation and aspiration according to strict clinical procedures. Informed consents were provided by all participants. This study was approved by the Research and Ethics Committee of the Shaoxing women and children hospital in Zhejiang, China. The inclusion criteria used for ERM patients were as follows: gestation age were between 6 and 10 weeks, two or more recurrent pregnancy losses. The control group was no obstetric complications or history of miscarriages and have at least one child. The detailed patient characteristics are presented in Table 1. All the samples were stored at −80 °C until used.

2.2. Protein extraction and digestion

A total of 1 mg of each placental villous sample was ground into a powder in liquid nitrogen and homogenized in extraction buffer (4% SDS, 1 mM DTT, 150 mM Tris-HCl, pH 8.0). Protein extraction was performed by cell lysis at 95 °C for 5 min, followed by sonication on ice. The crude extract was then incubated at 95 °C again and cleared by centrifugation at 14,000*g* for 30 min at 15 °C. Thereafter, the supernatant was collected and the protein concentration was measured by the BCA protein assay reagent from Pierce (Pierce, Rockford, IL, USA).

Protein digestion was performed as FASP protocol and was previously described [11,12]. Briefly, 200 μg of total-protein samples were diluted in 30 μL 4% SDS, 100 mM Tris-HCl pH 8.0, and 100 mM dithiothreitol solution, and were heated at 95 °C for 5 min. After each sample was cooled to room temperature, the sample was loaded onto an ultrafiltration filter (cutoff 10 kDa, Sartorius, Goettingen, Germany). We added 200 μL UT buffer (8 M Urea and 150 mM Tris-HCl, pH 8.0) to the filter and centrifuged it at 14,000*g* at 20 °C for 30 min. Subsequently, 100 μL of iodoacetamide solution (50 mM iodoacetamide in UT buffer) was added for blocking reduced cys-

Table 1
Clinical data of the patient groups.

	Sample name	Maternal age (y)	Gestational age (wk)	Maternal weight (kg)	Maternal height (cm)	Maternal BMI	Maternal blood pressure (mmHg)	Number of consecutive abortion experience
Control	C1	33	9	52	155	21.64	104/62	0
	C2	30	8	48	158	19.23	114/71	0
	C3	32	6	67	163	25.22	129/81	0
	C4	28	10	51	161	19.68	96/66	0
	C5	27	8	57	161	21.99	112/60	0
	C6	22	8	62	158	24.84	127/79	0
	C7	31	7	54	156	22.19	119/69	0
	C8	34	9	49	160	19.14	95/62	0
Early Recurrent miscarriage (ERM)	T1	32	9	47	156	19.31	112/68	4
	T2	29	9	62	164	23.05	133/84	2
	T3	31	8	44	155	18.31	98/62	3
	T4	28	11	60	162	22.86	118/77	2
	T5	33	8	53	158	21.23	115/69	2
	T6	34	8	57	164	21.19	123/74	3
	T7	27	10	61	155	25.39	132/79	2
	T8	33	9	48	162	18.29	95/61	2

teines, and, the samples were incubated for 20 min in darkness. Then the filters were centrifuged at 14,000*g* at 20 °C for 20 min. The filters were washed with 100 μL UT buffer at 14,000*g* for 20 min. This step was repeated 2 times. Then, 100 μL dissolution buffer (AB Sciex, Framingham, MA, USA) was added to the filter and it was centrifuged at 14,000*g* at 20 °C for 30 min, and, this step was repeated twice. Finally, 40 μL of trypsin (Promega, Madison, WI, USA) buffer (2 μg trypsin in 40 μL dissolution buffer) were added, and, the samples were digested overnight at 37 °C. Each filter unit was transferred to a new tube and centrifuged at 14,000*g* at 20 °C for 30 min. The resulting peptide concentrations were estimated by UV light spectral density at OD_{280}.

2.3. iTRAQ labeling

The resulting peptide mixture was labeled using the 8-plex iTRAQ reagent according to the manufacturer's instructions (AB Sciex, Framingham, MA, USA). Four placental villous tissue from the control group (C) were labeled with mass 113, 114, 115 and 116 isobaric iTRAQ tags, while the other four placental villous tissue from the RM group were labeled with mass 117, 118, 119 and 121 isobaric iTRAQ tags. The labeling solution was incubated at room temperature for 2 h before further analysis.

2.4. Strong cationic-exchange chromatography fractionation

Strong Cationic-exchange Chromatography Separation was performed as previously described method [11,12]. The combined sample was acidified with 1% trifluoroacetic acid before being subjected to strong cationic-exchange chromatography (SCX) fractionation using a PolySULFOETHYL column (4.6 × 100 mm, 5 μm, 200 Å, Poly LC Inc., Columbia, MD, USA). Solvent A consisted of 10 mM KH_2PO_4 in 25% (v/v) ACN, and, solvent B was solvent A with 500 mM KCl added. The solvents were applied using a gradient of 0%–10% solvent B for 2 min, 10–20% solvent B for 25 min, 20%–45% solvent B for 5 min, and 50%–100% solvent B for 5 min. The elution was monitored by absorbance at 214 nm, and fractions were collected every 1 min. Finally, these samples were combined into 10 fractions based on quantity of peptide and then desalted on C18 cartridges (Sigma, Steinheim, Germany). Each SCX salt step fraction was dried in a vacuum centrifuge and reconstituted with 40 μL 0.1% (v/v) trifluoroacetic acid.

2.5. LC - ESI- MS/MS analysis

LC - ESI- MS/MS Analysis was performed as previously described method [11,12].5 μg of peptide mixture from each fraction was subjected to nano LC-MS/MS analysis. Peptide mixtures were loaded onto the Thermo EASY-nLC column (Thermo Finnigan, San Jose, CA, USA) (100 mm × 75 μm, 3 μm) in solvent C (0.1% Formic acid) and separated with a linear gradient of solvent D (80% acetonitrile with 0.1% (v/v) formic acid) at a flow rate of 300 nL/min over 120 min: 0–100 min with 0%–45% solvent D; 100–108 min with 45%–100% solvent D; 108–120 min with 100% solvent D.

The Q-Exactive (Thermo Finnigan, San Jose, CA, USA) mass spectrometer acquired data in the positive ion mode, with a selected mass range of 300–800 mass/charge (m/Z). Dynamic exclusion was used with 40.0 s duration. Q-Exactive survey scans were set as 70,000 at m/z 200 and 17,500 at m/z 200 of resolution for HCD spectra. MS/MS data were acquired using a data-dependent acquisition method using the top 10 most abundant precursor ions. The normalized collision energy was 30 eV and the underfill ratio was defined as 0.1% on the Q-Exactive.

2.6. Protein Identification and Quantification

Protein Identification and Quantification was performed as previously described method [11,12]. Protein identifications were performed using the MASCOT search engine (version 2.2.1; Matrix Science, London, UK) embedded into Proteome Discoverer 1.3 (Thermo Electron, San Jose, CA, USA), searching against the Uniport database of human protein sequences (03-2013, 133,549 entries, downloaded from: http://www.uniprot.org) and the decoy database. Search parameters were set as follows: monoisotopic mass, peptide mass tolerance at ±20 ppm and fragment mass tolerance at 0.1 Da, trypsin as the enzyme and allowing up to two missed cleavages. Variable modifications were defined as oxidation of methionine and iTRAQ 8-plex labeled tyrosine while lysine and N-term of peptides labeled by iTRAQ 8-plex and carbamidomethylation on cysteine were specified as fixed modifications. False discovery rate (FDR) of both proteins and peptides identification as set to be less than 0.01. Protein identification was supported by at least one unique peptide identification.

2.7. Bioinformatics analysis

These significant differentially expressed proteins (p-value<0.05) were further inspected and the ones with differential expression ratio of over ± 1.3 were retained. The capability of the resulting differentially expressed proteins (DEPs) in differentiating two groups of samples was then evaluated by hierarchical cluster analysis. For this purpose, the Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) and the Java Treeview software (http://jtreeview.sourceforge.net) were used.

Downstream Effects Analysis, Upstream Regulator Analysis and network generation were performed using Ingenuity Pathway Analysis software package (QIAGEN, Redwood 185 City, CA). IPA is a knowledge database relying on published literature related to protein function, localization, relevant interactions and biological mechanisms. For the IPA, we performed the analysis using the profile for individual samples. Downstream Effects Analysis enables people to quickly visualize biological trends in experiment and predict the effect of gene expression changes in dataset on biological processes and disease or on toxicological functions. Use IPA Upstream Regulator Analysis to identify the upstream regulators that may be responsible for gene expression changes observed in experimental dataset. IPA predicts which upstream regulators are activated or inhibited to explain the up-regulated and down-regulated genes observed in your dataset. Knowledge of this regulatory cascade can help to understand the biological activities occurring in the tissues or cells that are studying. Calculated the z-score can infer the activation states (“activated” or “inhibited”) of implicated biological processes. Fisher's exact test was used to calculate a p-value to determine the probability that the association between proteins in the dataset, and, the biological process could be explained by chance alone.

2.8. Western blot

For western blot analysis, four protein samples of human placental villous tissue were prepared from control groups and four samples from ERM groups, separately. Human placental villous tissue was homogenized in 500 μL 1×RIPA buffer containing protease inhibitors (1 μg/mL leupeptin and 1 μg/mL phenylmethylsulfonyl fluoride). Samples were loaded at 30 μg/lane and separated in a 10% SDS gel.

The separated samples were transferred to a nitrocellulose transfer membrane (Bio-Rad, Hercules, CA, USA). After incubating for 1 h with blocking buffer, the membrane was incubated overnight at 4 °C with primary antibodies against Angiotensinogen (AGT) (Abcam ab97381, Cambridge, UK; 1:1000), MAPK14 (Beyotime, Beijing, China, 1:1000), Prothrombin (F2) (Abcam ab208589, Cambridge, UK; 1:1000) and β-Actin (Santa Cruz Biotechnology sc1616, Santa Cruz, CA, 1:1000) at 4 °C overnight. After three washes with 1×TBST, pH 7.4, each membrane was incubated with the appropriate secondary antibody (1: 1000) at room temperature for 1 h. After additional three washes, protein intensities were visualized with the enhanced ECL detection system (Beyotime, Beijing, China).

2.9. qPCR analysis

Total RNA was extracted with TRIzol (Invitrogen, CA) according to manufacturer's instructions. Total RNA from each sample was reverse-transcribed to cDNA using the PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan) and qRT-PCR was performed using the SYBR Premix Ex Taq (TaKaRa, Tokyo, Japan) and miRNA-specific primers for miR- (Ribobio, Guangzhou, China). The relative microRNA levels were normalized to U6 expression for each sample. Analyses of gene expression was performed by the $2^{-\triangle\triangle Ct}$ method.

2.10. Statistical analysis

Statistical analysis was carried out using Prizm 6 from GraphPad Software (San Diego, CA). Statistical significance for comparison between groups was determined by using Student's t-test. All samples were tested in triplicate, and the data are expressed as means ± SD? These differentially expressed proteins (p-value<0.05 and fold change over ± 1.3) were considered significant.

3. Results

3.1. Protein profiles of placental villous tissue

In this work, we used high accuracy LC-MS/MS to quantitatively detect and map proteins in human placenta villous specimens of the ERM and control groups. Using iTRAQ technology, we identified 2805 non-redundant proteins (Additional file 1: Table S1) in human placenta villous with high confidence (one or more unique peptides with an FDR less than 1%). The detailed information of the identified peptides is shown in Table S2 (Additional file 2). Among them, 314 proteins were found to be significantly different between the two groups (≥1.3-fold, Student's t-test, $p < 0.05$) (Additional file 1: Table S3), 209 proteins showed the increased expression while 105 proteins showed decreased expression. The Hierarchical clustering of these differentially expressed proteins is visualized in a heat map (Fig. 1A). The top 10 down-regulated and up-regulated proteins list in Table 2.

3.2. Western blot validation

After the iTRAQ analysis, we performed western blot analysis to verify the key proteomic differences discovered by iTRAQ analysis. Because the differentially expressed proteins were related with early development, we mainly analyzed the association of these proteins with the early embryonic development functions. We confirmed that the significantly altered proteins included Angiotensinogen (AGT), MAPK14 and Prothrombin (F2), which could affect embryonic development function. As shown in Fig. 1B, the western blot results were essentially in agreement with iTRAQ results.

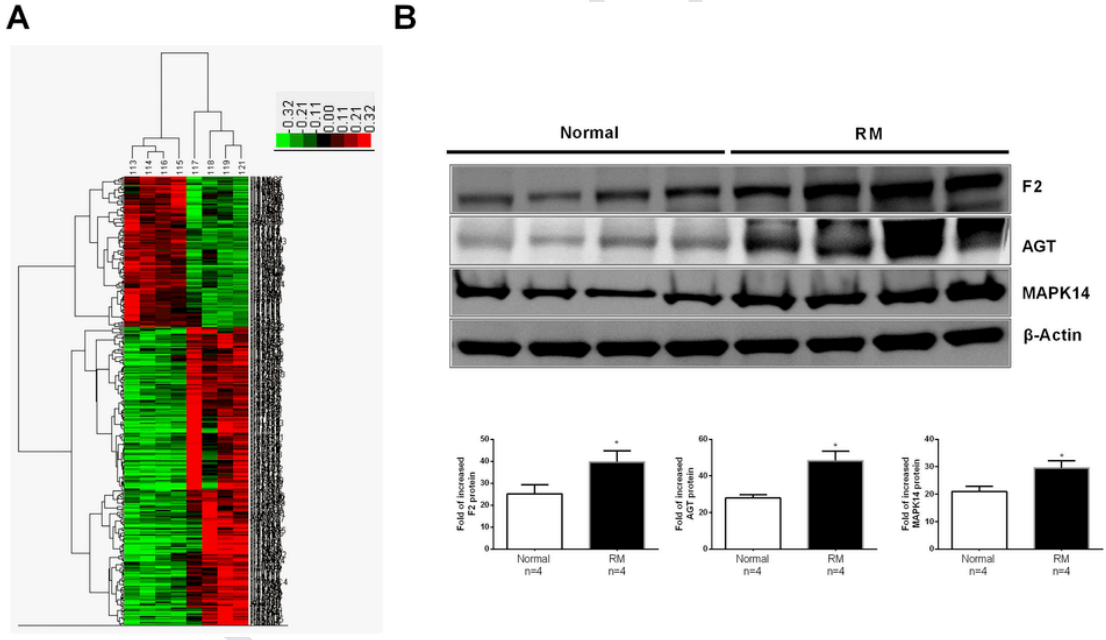


Fig. 1. Cluster analysis and western blot validation. (A) Hierarchical clustering of significant differentially expressed proteins in placental villous tissue. The bright red color indicates up-regulation, the deep green color indicates down-regulation, and black portrays no change. (B) Validation of the differential expression of three selected proteins by Western blot analysis: Angiotensinogen (AGT), MAPK14 and Prothrombin (F2). Data are present as mean ± SD (n = 4). $p < 0.05$ compared to the corresponding controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The top 10 down-regulated and up-regulated proteins identified from iTRAQ analysis.

UniProt Accession	Gene symbol	Name	MW [kDa]	calc. pI	*p*-value	Fold change
Q96HR9	REEP6	Receptor expression-enhancing protein 6	20.72	8.56	0.0001	−2.47
Q5QJE6	DNTTIP2	Deoxynucleotidyltransferase terminal-interacting protein 2	84.42	6.16	0.0136	−1.82
Q14978	NOLC1	Nucleolar and coiled-body phosphoprotein 1	73.56	9.47	0.0024	−1.73
Q9BY50	SEC11C	Signal peptidase complex catalytic subunit SEC11C	21.53	9.20	0.0056	−1.64
P84103	SRSF3	Serine/arginine-rich splicing factor 3	19.32	11.65	0.0003	−1.61
Q14739	LBR	Lamin-B receptor	70.66	9.36	0.0001	−1.60
P15954	COX7C	Cytochrome *c* oxidase subunit 7C, mitochondrial	7.24	10.27	0.0027	−1.59
Q13247	SRSF6	Serine/arginine-rich splicing factor 6	39.56	11.43	0.0001	−1.58
P01215	CGA	Glycoprotein hormones alpha chain	13.07	8.19	0.0001	−1.57
P98095	FBLN2	Fibulin-2	126.49	4.82	0.0046	−1.55
P04004	VTN	Vitronectin	54.27	5.80	0.0032	2.17
P01019	AGT	Angiotensinogen	53.12	6.32	0.0209	2.20
P02654	APOC1	Apolipoprotein C-I	9.33	8.47	0.0094	2.29
P43003	SLC1A3	Excitatory amino acid transporter 1	59.53	8.41	0.0139	2.30
Q9Y3E0	GOLT1B	Vesicle transport protein GOT1B	15.42	10.36	0.0123	2.33
P51888	PRELP	Prolargin	43.78	9.38	0.0191	2.38
P01242	GH2	Growth hormone variant	24.98	7.71	0.0219	2.43
Q9BV10	ALG12	Dol-P-Man:Man(7)GlcNAc(2)-PP-Dol alpha-1,6-mannosyltransferase	54.62	9.58	0.0043	2.66
Q8NGA1	OR1M1	Olfactory receptor 1M1	34.82	8.91	0.0316	3.33
Q9C005	DPY30	Protein dpy-30 homolog	11.24	4.88	0.0006	3.46

3.3. Bioinformatics analysis of differentially expressed proteins

3.3.1. Downstream Effects Analysis

The MS identified proteins which were confirmed to change in expression were further analyzed by Ingenuity Pathway Analysis software (IPA, www.ingenuity.com) (Qiagen, Germany). The IPA generated a rank order of significance of association of the differentiated proteins, and the proteins listed by the software were involved in "Disease and Disorder", and in "Physiological System Development and Functions". According to overlapping *p*-values, these differentially expressed proteins were significantly related to 26 subcategories of "Disease and Disorder" (Additional file 4: Fig. S1), and, 24 subcategories of "Physiological System Development and Functions" (Additional file 5: Fig. S2).

Accordingly, based on "Disease and Disorder" analysis, 30 proteins were related to Developmental Disorder (Fig. 2), and 57 proteins were related to Reproductive System Disease. Based on "Physiological System Development and Functions" analysis, 33 proteins were described to be associated with Embryonic Development (Fig. 3), especially in the growth of embryo.

3.3.2. Upstream Regulator Analysis

The term "upstream regulator" as used in IPA refers to any molecule that can affect the expression of another molecule. Upstream regulators cover the gamut of molecule types founds in the literature, from transcription factors, to cytokines, microRNAs, receptors, kinases, chemicals and drugs. In our study, based on 33 proteins which were related with Embryonic Development, the upstream regulator were miR-615, miR-637, miR-1275, beta-estradiol, dexamethasone, doxorubicin and lipopolysaccharide (Fig. 4A), we used qPCR analysis to valified the expression of these three miRNAs (Fig. 4B).

3.3.3. Network analysis

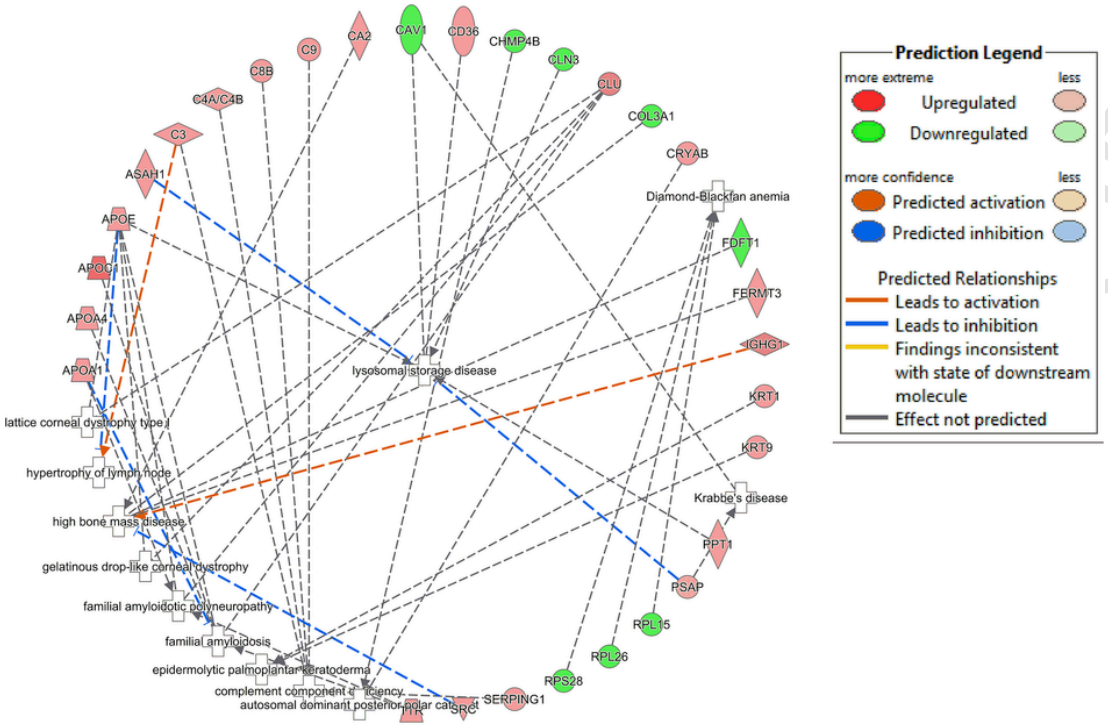
To better understand these differentially expressed proteins, the data were further analyzed using network analysis. Hypothetical networks were built among the experimental proteins and the IPA database proteins. The most related network emerged comprising 30 of those differentially expressed proteins (DEPs). It is associated with the IPA functions "Developmental Disorder", the core factors are Angiotensinogen (AGT), MAPK14 and Prothrombin (F2) (Fig. 5), these three proteins are also related with early embryonic development.

4. Discussion

Proteins that perform biological functions directly are rich in information that has been extremely valuable for the description of biological processes. The correlation between mRNA/DNA and protein levels is insufficient to predict protein expression levels [13]. Proteomics has many advantages compared with other technology. Mass spectrometry (MS) allows the multivariate analysis of complex patterns of new biomarkers without knowledge of the human proteome could help fill the gap between genomes and phenotypes, transforming the way we develop diagnostics and therapeutics, and thereby enhancing overall biomedical research and future healthcare [14].

The placenta is a major contributor to pregnancy, and many pregnant women suffer miscarriage during early gestation, which has an adverse effect on the quality of life worldwide. Despite the considerable research efforts expended to understand the pathogenesis of the early recurrent miscarriage (ERM), few significantly advances have been made in recent decades because of the complexities of early development. Thus, the identification of proteins with different expression profiles related to early recurrent miscarriage (ERM) is essential to understand the pathophysiological mechanism. The robust and high-throughput of mass spectrometry-based proteomics is suited to clinical applications [15]. iTRAQ (isobaric tag for relative and absolute quantitation) is a powerful quantitative proteomics technique for the identification and characterization of protein expression pattern. In this study, we provide unique insight into differentially expressed proteins of placenta villous specimens from early recurrent miscarriage (ERM) subjects via iTRAQ based technology; our data is useful for obtaining molecular insights into early pregnancy loss.

Using IPA, we performed core analysis of the differentially abundant proteins. IPA includes a manually annotated database of protein interactions and metabolic reactions obtained from the scientific literature. Based on our proteomic analysis, we first analysis diseases and functions of these differentially expressed proteins (DEPs). In "physiological system development and function" analysis, we found that 33 DEPs are related with "Embryonic Development", further

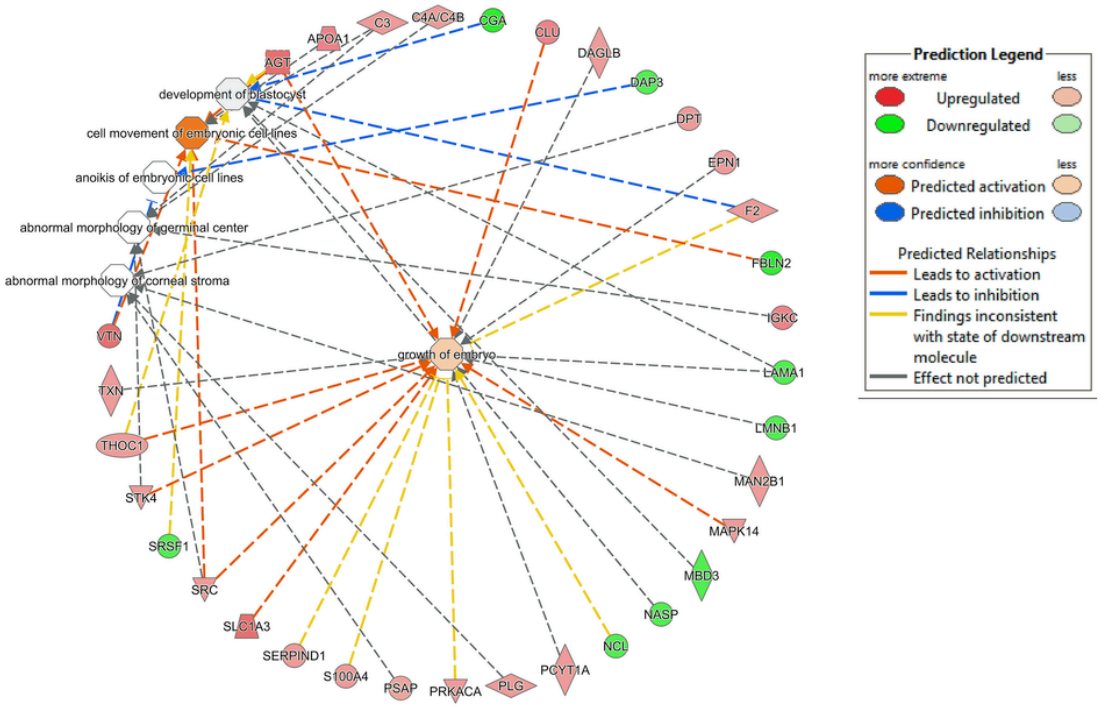


Diseases or Functions Annotation	*p*-Value	Molecules	Molecules Number
lysosomal storage disease	4.78E-04	APOE,ASAH1,CAV1,CD36,CLN3,PPT1,PSAP	7
familial amyloidosis	1.75E-05	APOA1,APOA4,APOC1,APOE,CLU,TTR	6
high bone mass disease	4.86E-04	CA2,COL3A1,FDFT1,FERMT3,IGHG1,SRC	6
complement component deficiency	3.06E-06	C3,C4A/C4B,C8B,C9,SERPING1	5
familial amyloidotic polyneuropathy	1.83E-05	APOA1,APOE,CLU,TTR	4
Diamond-Blackfan anemia	1.40E-03	RPL15,RPL26,RPS28	3
epidermolytic palmoplantar keratoderma	2.02E-04	KRT1,KRT9	2
hypertrophy of lymph node	6.02E-04	APOE,C3	2
lattice corneal dystrophy type I	6.02E-04	APOE,CLU	2
Krabbe's disease	1.19E-03	CAV1,PSAP	2
gelatinous drop-like corneal dystrophy	1.19E-03	APOE,CLU	2
autosomal dominant posterior polar cataract	1.97E-03	CHMP4B,CRYAB	2

Fig. 2. Disease analysis. Disease analysis of specific differentially expressed proteins associated with "Developmental Disorder" by the iterative Ingenuity Pathway Analysis software program. These detail informations are listed in the table. For this developmental disease network, genes or gene products are represented as *nodes*, and the biological relationship between two nodes is represented as an *edge*. All edges are supported by at least one publication as stored in the Ingenuity Knowledge database. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analysis show that "growth of embryo" are the most related function, about 19 differentially expressed proteins are involved. In these proteins, AGT affects embryonic development through Renin- Angiotensin system [16]. MAPK14 involved in Mitogen-activated protein kinase (MAPK) pathways which mediate embryonic responses and apoptosis [17]. Defects in prothrombin (F2) are a cause of susceptibility to thrombosis (THR), thrombosis can cause recurrent miscarriage [18]. Lamin B1 is required for mouse development and nuclear integrity [19]. NASP is required for preimplantation development into the blastocyst stage in cattle [20]. Parietal endoderm secreted S100A4 promotes early cardiomyogenesis in embryoid bodies [21]. Thoc1/Hpr1/p84 is essential for early embryonic development in the mouse [22]. Txn plays an essential role during embryogenesis in most developing tissues [23]. VTN promote endoderm differentiation of hESCs [24]. Therefore, the abnormal expression of these proteins may related with embryonic development. However, the precise relationship of these proteins with early recurrent miscarriage (ERM) need further investigation.

Use IPA Upstream Regulator Analysis to identify the upstream regulators that may be responsible for gene expression changes observed in experimental dataset. IPA predicts that which upstream regulators are activated or inhibited to explain the up-regulated and down-regulated genes observed in dataset. In the upstream analysis of "Embryonic Development", the upstream regulator were miR-615, miR-637, miR-1275, beta-estradiol, and lipopolysaccharide (Fig. 4). miR-615, miR-637 and miR-1275 inhibited these related proteins, while beta-estradiol and lipopolysaccharide activated these expression. Beta-estradiol affects embryonic development in many species, including American alligator [25], zebrafish [26], chick [27,28], Japanese medaka [29], brown trout [30]. Doxorubicin blocked pre-implantation development in early mouse embryos by altering poptosis-related gene expression and inactivating DNA repair by PARP [31]. LPS challenge could to a suboptimal environment for embryo devel-



Diseases or Functions Annotation	*p*-Value	Molecules	Molecules Number
growth of embryo	1.84E-03	AGT,CLU,DAGLB,EPN1,F2,LAMA1,LMNB1,MAPK14,NASP,NCL, PCYT1A,PRKACA,S100A4,SERPIND1,SLC1A3,SRC,STK4,THOC1,TXN	19
cell movement of embryonic cell lines	1.19E-03	AGT,APOA1,C3,FBLN2,SRC,SRSF1,VTN	7
development of blastocyst	1.45E-03	AGT,CGA,F2,LAMA1,MBD3,NASP,THOC1	7
abnormal morphology of germinal center	7.13E-04	C3,C4A/C4B,IGKC,SRC,STK4	5
abnormal morphology of corneal stroma	1.06E-04	DPT,MAN2B1,PLG,PSAP	4
anoikis of embryonic cell lines	6.02E-04	DAP3,VTN	2

Fig. 3. Functional analysis. Functional analysis of specific differentially expressed proteins associated with "Embryonic Development" by the iterative Ingenuity Pathway Analysis software program. These detail informations are listed in the table. For this developmental function network, genes or gene products are represented as *nodes*, and the biological relationship between two nodes is represented as an *edge*. All edges are supported by at least one publication as stored in the Ingenuity Knowledge database. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

opment [32]. However, these microRNAs have little study in early recurrent miscarriage (ERM). Upstream analysis can provide us clues for future study. Therefore, further study is needed to clarify the roles of these proteins in early embryonic development of recurrent miscarriage.

Authors' roles

H-G.D, M.F, B.Y, Q-Q.F, B.L and H-G.C prepared samples. H-T.P and Y.C performed LC-MS/MS analysis. H-T.P, Y-J.T, X. J and X-Q.X analyzed the data, Z.T, H-G.D, M.F and H-T.P designed and conceived the study. All authors read and approved the final manuscript.

Conflicts of interest

None declared.

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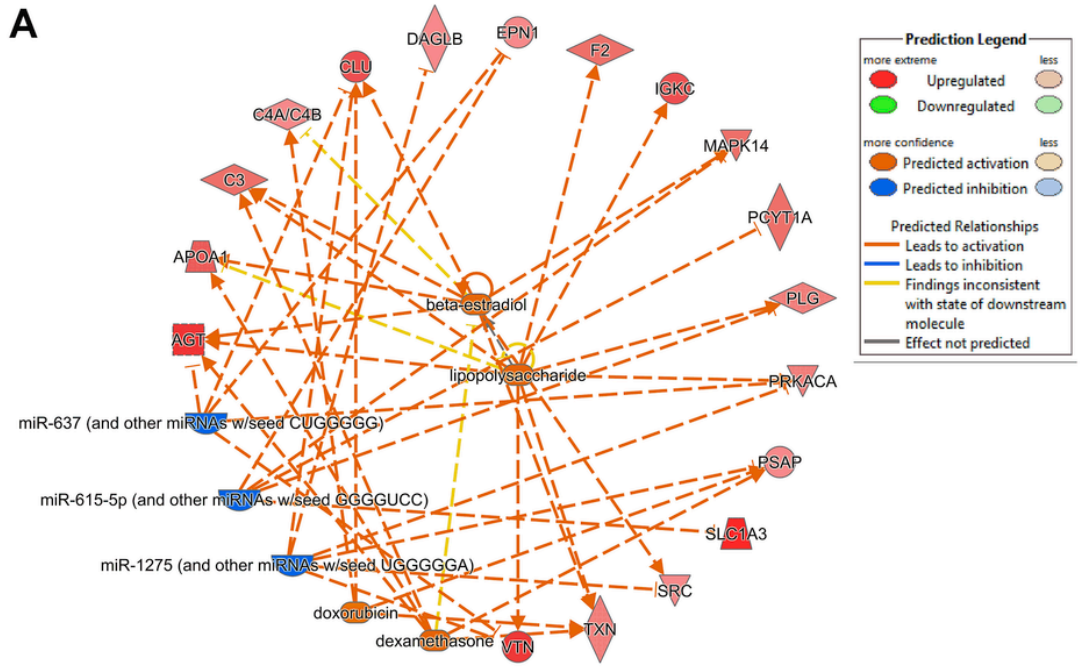
Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.placenta.2017.11.001.

Uncited reference

[33].



Upstream Regulator	Predicted Activation State	Activation z-score	*p*-value of overlap	Target molecules in dataset
beta-estradiol	Activated	2.074	2.62E-04	AGT,APOA1,C3,C4A/C4B,CLU,MAPK14,SRC,TXN
dexamethasone	Activated	2.189	2.71E-02	AGT,APOA1,C3,PSAP,TXN
doxorubicin	Activated	2.000	5.80E-04	C4A/C4B,CLU,PSAP,TXN
lipopolysaccharide	Activated	2.217	2.21E-05	AGT,APOA1,C3,F2,IGKC,PLG,PRKACA,TXN,VTN
miR-637	Inhibited	-2.207	2.82E-02	AGT,CLU,EPN1,PRKACA,VTN
miR-615-5p	Inhibited	-2.201	8.13E-03	EPN1,MAPK14,PCYT1A,PLG,SLC1A3
miR-1275	Inhibited	-2.434	1.03E-02	CLU,DAGLB,PRKACA,PSAP,SRC,VTN

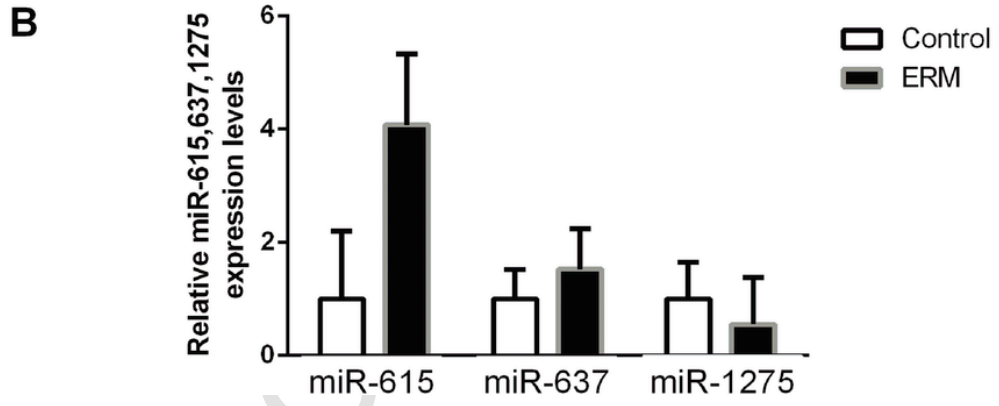
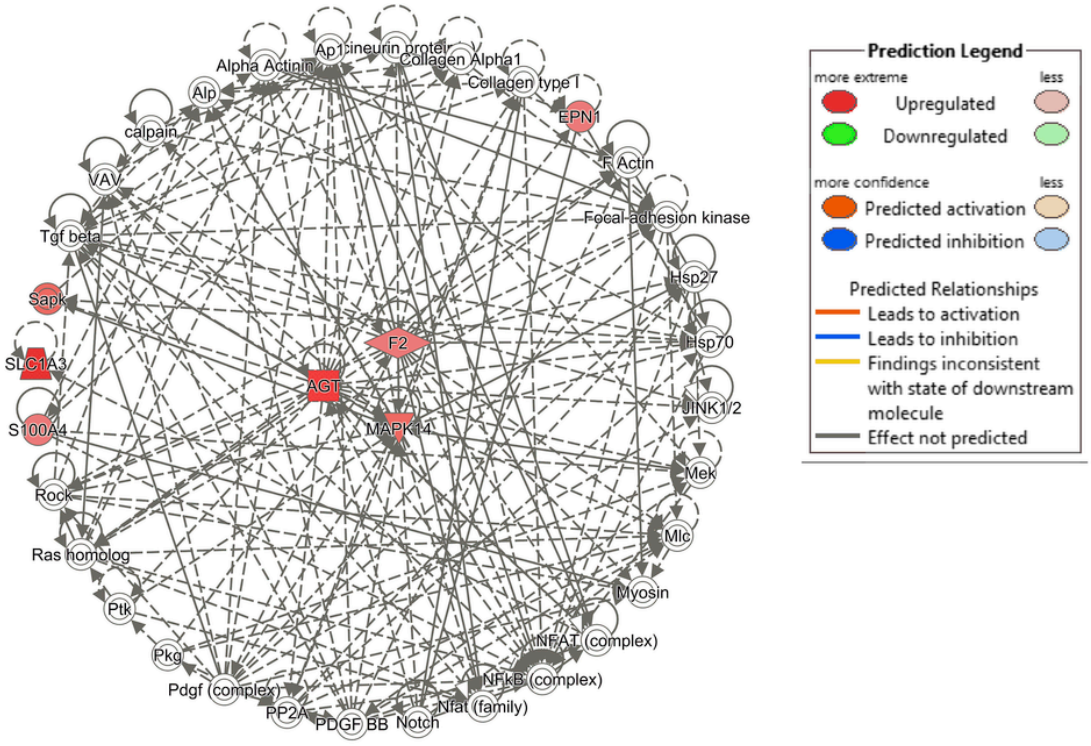


Fig. 4. Upstream analysis. (A) Upstream analysis by IPA based on 33 differentially expressed proteins related with "Embryonic Development". These detail informations are listed in the table. Colorized nodes represented our input proteins. Green: down-regulated proteins. Red: up-regulated proteins. **(B)** Relative miR-615,637,1275 expression levels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



ID	Score	Focus Molecules	Top Diseases and Functions
1	54	30	Cardiovascular Disease, Developmental Disorder, Hematological Disease
2	39	24	Hematological System Development and Function, Molecular Transport, Organismal Functions
3	32	21	RNA Post-Transcriptional Modification, Cellular Assembly and Organization, Tissue Morphology
4	30	20	Cellular Assembly and Organization, Cell Death and Survival, Nervous System Development and Function
5	30	20	Metabolic Disease, Neurological Disease, Psychological Disorders

Fig. 5. Network analysis. Network that emerged was "Developmental Disorder". These detail informations are listed in the table. The relevant pathway maps were then prioritized according to their statistical significance ($p < 0.001$), and networks were graphically visualized as hubs (proteins) and edges (the relationship between proteins). Colored nodes represent our input proteins. Green: down-regulated proteins; red: up-regulated proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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