Screening and identification of endometrium proteins as novel potential biomarkers for repeated implantation failure

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Repeated implantation failure (RIF) patients are the bottleneck of clinical pregnancy rate for assisted reproductive technology, due to poor endometrial receptivity. We collected endometrial tissue at the window of implantation by hysteroscopybiopsy during September 2016 to December 2019, clinical data were collected as well. A total of 82 differentially expressed endometrium proteins were identified, including 55 up-regulated proteins (>1.50-fold, $P<0.05$) and 27 down-regulated proteins (<0.67-fold, $P<0.05$) by iTRAQ labeling coupled with 2D LC MS/MS technique. String analysis found interactions between these proteins which assembled in two bunches, ribosomal proteins and blood homeostasis proteins. In addition, the most significant enriched Gene Ontology terms were negative regulation of hydrolase activity, blood microparticle, and enzyme inhibitor activity. Verified results highlighted the corticostroid binding globin as the specific protein of endometrial receptivity, representing low progesterone level. Our study provided experimental data to establish the objective indicator of endometrial receptivity, and also provided a new sight into the pathogenesis of RIF.

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Abstract

Repeated implantation failure (RIF) patients are the bottleneck of clinical pregnancy rate for assisted reproductive technology, due to poor endometrial receptivity. We collected endometrial tissue at the window of implantation by hysteroscopybiopsy during September 2016 to December 2019, clinical data were collected as well. A total of 82 differentially expressed endometrium proteins were identified, including 55 up-regulated proteins (>1.50-fold, $P<0.05$) and 27 down-regulated proteins (<0.67-fold, $P<0.05$) by iTRAQ labeling coupled with 2D LC MS/MS technique. String analysis found interactions between these proteins which assembled in two bunches, ribosomal proteins and blood homeostasis proteins. In addition, the most significant enriched Gene Ontology terms were negative regulation of hydrolase activity, blood microparticle, and enzyme inhibitor activity. Verified results highlighted the corticostroid binding globin as the specific protein of endometrial receptivity, representing low progesterone level. Our study provided experimental data to establish the objective indicator of endometrial receptivity, and also provided a new sight into the pathogenesis of RIF.

Introduction

According to the World Health Organization, infertility prevalence was highest in South Asia, Sub-Saharan Africa, North Africa/Middle East, and Central/Eastern Europe and Central Asia (Mascarenhas et al., 2012). The infertility rate of China's childbearing age couples was 25% (Zhou et al., 2018). Assisted reproductive technology (ART) is the best way to help infertility patients to have children successfully at this stage, and the number of these patients increased randomly (Kushnir et al., 2017).

In vitro fertilization and embryo transfer (IVF-ET) is the most common way of ART. Although it can help some infertile women to get pregnant successfully, there were still some patients who could not get pregnant even after multiple transplantations (2-6 times) of high-quality embryos, whom were classified as repeated implantation failure (RIF) patients (Margalioth et al., 2006). These patients are the bottleneck of clinical pregnancy rate of ART technology. The two key factors for successful implantation are embryo quality and endometrial receptivity. The embryo quality nowadays could be evaluated by several criteria. Meanwhile, endometrial receptivity is defined as the period during which the endometrial epithelium acquires functional, but transient, ovarian steroid-dependent status supportive to blastocyst acceptance and implantation. This period was called window of implantation (WOI). The golden standard for evaluating endometrial receptivity is the pinopodes in endometrial histology. However, it changes from samples and menstrual cycles (Acosta et al., 2000). The ultrasound examination was a noninvasive and inexpensive test widely used, which contained endometrial thickness, endometrial type, endometrial volume, uterine artery and sub-endometrial blood flow. However, this examination with strong subjectivity shows a little effect in predicting pregnancy rate with single parameter. A number of studies have applied proteomic techniques to analyze human endometrium crossing different cycle phases, sampled by either biopsy or curettage (Chen et al., 2009; Yap et al., 2011). Former researchers (Yap et al., 2011) identified IL-11 regulated plasma membrane proteins ANXA2, and the lipid-raft protein FLOT1 in human endometrial epithelial cells in vitro in the receptive phase of the menstrual cycle.

In this study, differential WOI endometrium proteins were screened using iTRAQ labeling coupled with 2D LC-MS/MS technique in order to find potential biomarkers for RIF patients. Our study provided experimental data to establish the objective indicator of endometrial receptivity, and also provided a new way to reveal the pathogenesis of RIF.

Materials & Methods

Sample collection

This study was approved by the Ethics Committee of the Faculty of Medicine (Hangzhou Women's Hospital, China) (Approval Number: 2016001-10). Written informed consents were obtained from all subjects before endometrium collection.

RIF was defined as failure of three or more cycles in which reasonably high-quality embryos were transferred (Margalioth et al., 2006). The criteria of embryo quality were determined as grade I and grade II were high-quality embryos (Gardner et al., 1998). The criteria of blastocyst quality were also determined according to Gardner et al. (Gardner et al., 1998). Blastocysts were considered high-quality if they had a grade 3 or 4 blastocoel, a grade A or B inner cell mass, and a grade A or B trophectoderm on days 5 or 6. Fifty-two RIF cases and 135 pregnant cases undergoing IVF-ET treatment were collected in our hospital during September 2016 to December 2019. Data including age, follow-up outcomes, and clinical examination findings were collated into databases.The endometrium tissue was collected at WOI by

hysteroscopybiopsy, each for 200～500 μg. We washed these tissues by saline immediately, then froze in liquid nitrogen and stored until protein extraction.

Endometrium protein extraction

We randomly selected 2 cases of pregnant patients and 3 cases of RIF patients for endometrial protein extraction. Protein extraction was performed using lysis buffer after grinded the sample to powder in liquid nitrogen. Add PMSF with final concentration of 1mM and EDTA with final concentration of 2mM, wait for 5 min, and then add DTT with final concentration of 10mM. Ice bath ultrasound for 5 min, the lysate was centrifuged at 15,000 g for 20 min. The supernatant was precipitated with 5 times volume of acetone and incubated at 20 °C for 2 h and then centrifuged at 15,000 g and 4 °C for 20 min. The precipitate was washed by chilled acetone, incubated at 20 °C for 30 min and centrifuged again at 15,000 g and 4 °C for 20 min. The washing was repeated two times. After airdried, the precipitate was re-dissolved with the lysis buffer. After ice bath ultrasound for 5 min, centrifuged precipitate again at 15,000 g and 4 °C for 20 min. The supernatant was determined using a Bradford Protein Assay Kit to detect the protein concentration.

Protein digestion and iTRAQ-2D LC-MS/MS

A total of 100 μg protein from each group was digested using trypsin (Promega, USA) at 1:50 trypsin-to-protein mass ratio at 37 °C for 16 h. Then peptides were reconstituted in 0.2M TEAB and processed according to the manufacturer's protocol for 8-plex iTRAQ reagent (AB SCIEX, Framingham, MA, USA). Three biological replicates of the RIF group were labeled with 113, 114 and 115 isobaric tags, respectively. The peptides with two biological replicates of pregnant group were labeled with 116 and 117 isobaric tags, respectively.

The peptides mixtures were subjected to the first-dimensional fractionation procedure using high pH reversed-phase chromatography column (Phenomenex, Gemini-NX 3u C18110A, 150*2.00mm). A total of 16 fractions were finally collected and dried for the following LC-MS analysis. The lyophilized peptide fractions were re-suspended in 2% acetonitrile containing 0.1% formic acid, and loaded into a C18 trap column (Acclaim PepMap 75μm × 150mm, C18, 3μm, 100A). The online Chromatography separation was per- formed on the nanoLC system (Dionex Ultimate 3000 RSLCnano). The trapping and desalting procedures were carried out at a flow rate of 3 μL/min for 5 min with 100% solvent A (0.1% formic acid, 2% acet- onitrile and 98% water). Then, Peptides were than eluted using 65 min gradient of buffer A (0.1% formic acid) to buffer B (80% ACN containing 0.1% formic acid) at 300 nL/min. It was used on an analytical column (Acclaim PepMap 75 μm × 15 cm C18-CL, 3 μm 100 Å, Thermo160321). IDA (information-dependent acquisition) mass spectrum technique was used to acquire tandem MS data on Q Exactive system (Thermo Scientific) fitted with a Nanospray ion source. Data were acquired using an ion spray voltage of 2.2 kV. MS spectra were acquired across the scan range of 350–1800 m/z in a resolution of 70,000 using maximum injection time (60 ms) per spectrum. Twenty most intense precursors per MS cycle were selected for fragmentation detected with 100 ms maximum injection time. Tandem mass spectra were recorded in a resolution of 17,500 with

rolling collision energy on and iTRAQ reagent collision energy adjustment on. For accurate mass measurements, the lock mass option was enabled. Dynamic exclusion was set for 10 s.

The MS/MS data were analyzed using IPeak and IQuant software to obtain the protein identification and quantification (Wen et al., 2015; Wen et al., 2014), and NCBI *human* genome. Only proteins identified at global FDR ≤ 1% with ≥ 1 peptide were considered for protein lists and further downstream analysis. To determine a differentially expressed protein, it must be identified and quantified with at least 1 significant peptide and the p-values of proteins quantitation should be less than 0.05 and fold change ≥ 1.5.

Bioinformatics analysis

Functional annotation was performed using Gene Ontology (GO) database (http://www.geneontology.org) including cellular component, molecular function, and biological process. The differentially expressed protein–protein network was analyzed by STRING software (http://www.string-db.org/).The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/or http://www.kegg.jp/) was used to predict the main metabolic pathways (Kanehisa et al., 2007). We obtained the significantly enriched GO/pathway items by hypergeometric test. The EggNOG database (http://eggnogdb.embl.de) was used for pairwise orthology predictions, functional annotation and classification (Huerta-Cepas et al., 2015).

Westernblot analysis

Tissues were lysed with 200 μL of RIPA lysate (P0013B, Beyotime, Shanghai, China) plus 1mM PMSF at 4 °C for 30min. After centrifugation at 12,000 rpm for 10 min at 4 °C, the supernatant was harvested and stored at -80 ℃. BCA method was used to determine protein concentration using BCA quantitative kit (P0009, Beyotime). Protein samples were subjected to polyacrylamide gel electrophoresis, which were then transferred onto PVDF membrane (IPVH00010, Millipore, Massachusetts, USA). The membrane was blocked by 5% skimmed milk powder at room temperature for 2h. Then, the membrane was incubated with primary antibodies including cortisol binding globulin (ab110648, abcam, Cambridge, United Kingdom), Antithrombin III (ab126598, abcam) and GAPDH (60004-1-Ig, proteintech, Beijing, China) at 4 °C overnight, followed by goat anti-rabbit IgG-HRP (BK0027, BEST, Xian, China) and goat anti-mouse anti-rabbit IgG-HRP (BK0023, BEST) secondary antibodies at room temperature for 1.5h. The blots were visualized using ECL Plus Luminous Kit (S17851, Yeasen, Shanghai, China) with a gel imaging system, and the results were measured with Image J.

Statistical analysis

The parametric data were tested using the chi-square test for the composition ratios, paired t-tests for means of two groups. *P* values <0.05 were considered statistically significant by the SPSS software (Chicago, IL, version 18.0).

Results

Clinical data analysis

We recruited 52 RIF cases and 135 pregnant cases undergoing IVF-ET treatment during September 2016 to December 2019. Data including age, follow-up outcomes, and clinical examination findings were collated into databases. Considering elder age could affects the pregnancy rate, a total of 40 subjects with RIF, aged under 40 years old, and 80 paired subjects with pregnancy were finally analyzed. There were no significant differences between RIF patients and pregnant controls in general and clinical data (P>0.05, Table 1).

Endometrial proteomics results

We identified a total of 6,102 proteins through iTRAQ-2D LC-MS/MS. Among the 6,102 identified proteins, a total of 5,840 had GO annotations, a proportion of 95.71% of all proteins; a total of 5,504 had KEGG annotations, a proportion of 90.20% of all proteins; finally, a total of 6,097 had EggNOG annotations, a proportion of 99.92% of all proteins.

We performed quality control on the quantitative results through volcano maps and the distributions of Coefficient of Variation. The hierarchical clustering provided a visualized mode to display the clustering patterns of the different expressed proteins between the groups (Fig. 1). Further screening revealed 82 differentially expressed proteins in RIF patients compared with pregnant controls, including 55 up-regulated proteins (>1.50-fold, P<0.05) and 27 down-regulated proteins (<0.67-fold, P<0.05) (Table 2).

Bioinformatics analysis results

Gene Ontology analysis of differentially expressed proteins revealed that most of the proteins were involved in the response to stimulus (42 proteins), extracellular region (35 proteins), and structural molecule activity (10 proteins) (Fig. 2A). The most significant enriched GOs were negative regulation of hydrolase activity (SPB6, APOA1, GMIP, THBG, CBG, ANT3, and FETUA), blood microparticle (VTDB, IGHG4, APOA1, A1AG2, FETUA, ANT3, and A1AG1), and enzyme inhibitor activity (ANT3, FETUA, CBG, ASPN, SPB6, THBG, and APOA1) through hypergeometric testing (Figure 2B). Among them, ANT3, FETUA, and APOA1 repeated three times, while CBG and THBG repeated two times. The molecular function of these repeated proteins is serine-type endopeptidase inhibitor activity (Fig. 2C). Furthermore, String analysis found interactions between these proteins (Fig. 2D) which assembled in two bunches, ribosomal proteins and blood homeostasis proteins.

In addition, the KEGG pathway mapping revealed some significant pathways: immune system (7 proteins) and translation (5 proteins) (Fig. 3A), enriched in primary immunodeficiency (Fig. 3B). Finally, we analyzed through the EggNOG database that the differential proteins associated with RIF are mostly clustered in classifications such as posttranslational modification, protein turnover, chaperones (22 proteins), translation, ribosomal structure and biogenesis (8 proteins), and carbohydrate transport and metabolism (6 proteins) (Fig. 3C).

Westernblot results

We verified endometrium ATIII and CBG level by western blot, finding significant higher level of CBG in RIF patients (Fig. 4). Furthermore, a significant difference in CBG was found using grayscale detection by Image J (P=0.003).

Discussion

After more than 40 years, IVF technology has made rapid progress. However, the clinical pregnancy rate remained around 33.8-42.7% (European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE) et al., 2016; Sunderam et al., 2019). After the implementation of blastocyst transplantation, the clinical pregnancy rate increased to 60.4% (Ozgur et al., 2018). One of the key factors affecting the pregnancy rate of IVF is the RIF, actually the poor endometrial receptivity is the main reason for the decrease of pregnancy possibility in RIF patients. In our study, the endometriumof IVF-ET patients during WOI was collected and divided into RIF group and pregnant controls group according to pregnancy outcomes. Specific proteins related to endometrial receptivity were screened by iTRAQ-2D LC-MS/MS.

Through iTRAQ-2D LC-MS/MS and bioinformatics analysis, 82 differential proteins were obtained in the endometrium of RIF patients during the WOI, of which 55 were higher (> 1.50 times, $P< 0.05$) and 27 were lower (< 0.67 times, $P< 0.05$). The differential proteins obtained in this study have also been identified in the early proteomic studies. Hannan et al. obtained 7 differential proteins of the uterine lavage fluid of pregnant/non-pregnant patients in the WOI by 2D-DiGE, furthermore, the antithrombin III was significantly increased in the non-pregnant patients by immunohistochemistry (Hannan et al., 2010). In this study, the expression of antithrombin III was 1.77 times as much as that in the pregnant group, which was similar to the previous results, indicating the reliability of iTRAQ-2D LC-MS/MS. In addition, ribosomal protein and plastin are two kinds of proteins that have been screened both in this study and previous studies (Domínguez et al., 2009).

Through gene annotation, Gene Ontology classification and hypergeometric test, the most significant GO items were targeted in negative regulation of hydrolase activity, blood microparticle, and enzyme inhibitor activity (Fig. 2B). The mostly repeated ANT3 (P01008), FETUA (P02765), APOA1 (P02647), CBG (P08185), and THBG (P05543) assembled in serine-type endopeptidase inhibitor activity in GO terms, indicating that these genes are the key genes encoding endometrial receptive specific proteins.We have reason to believe that the molecular function of these genes is the key target site of endometrial receptivity.

Combining known biological functions and literature review, we choose ANT3 and CBG as candidate proteins for endometrial receptivity. Literature review found that the protein encoded by ANT3 is antihrombin-III, a representative protein of prethrombotic state. The prethrombotic state is one of the suspected causes of RIF (Qublan et al., 2006). Former study confirmed TGF-β3 is secreted by leiomyoma. And TGF-β3 can significantly reduce the expression level of ATIII in normal endometrial stromal cells, resulting in menorrhagia and reproductive dysfunction, which shows that the disorder of coagulation function is one of the main reasons for RIF (Sinclair et al., 2011). However, in our verification, ANT3 showed no significant difference in RIF patients and pregnant controls. These might due to the consist of our RIF patients, in our study, oviduct factors contain the most, not the leiomyoma.

CBG encodes corticosteroid-binding globulin, a multifaceted component in cortisol delivery, acute and chronic inflammation, and metabolism and neurocognitive function (Meyer et al., 2016). The increasing CBG level in pregnant period was also important at the materno-fetal interface (Lei et al., 2015). In our study, we found significant higher level of CBG in RIF patients (Fig. 4). Misao et al. suggested that the decrease of progesterone level in the blood can lead to the increase of CBG expression in endometrium (Misao et al., 1995). However, in our study, the lower level of serum progesterone was also found in RIF patients, but with no significant difference (Table 1). We believed a larger sample of research was needed.

KEGG analysis and enrichment analysis showed that differential proteins were related to the immune system and primary immunodeficiency (Fig. 3A, 3B).Therefore, the change of immune response in RIF patients is self-evident. It has been shown that the abnormal and functional defects of immune cells and molecules in endometrium during implantation can lead to pregnancy failure (Liu et al., 2016). Therefore, we suspect that the change of endometrial immune microenvironment could lead to RIF, which provides a new way for clinical assistant treatment for RIF patients. In the present study, most proteins were annotated with "posttranslational modification, protein turnover, chaperones" and "translation, ribosomal structure and biogenesis function", meaning that most proteins involved in modification after translation, synthesis and degradation, folding, maintenance, intracellular transport, as well as mRNA translation based on the EggNOG database except function unknown. The results of KEGG and String analysis also confirmed that translation is impacted.

Conclusions

In general, our study screened the endometrium proteomics of RIF patients through iTRAQ-2D LC-MS/MS, highlighted the corticostroid binding globin as the specific protein of endometrial receptivity. Our results provide experimental data to establish the objective indicator of endometrial receptivity, moreover, also give a new sight into the pathogenesis of RIF.

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Table 1(on next page)

General and clinical data of repeated implantation failure patients (RIF) and pregnant controls.

All data are presented asthe mean ± SD. BMI: body mass index; AMH: anti-Mullerian hormone. [a]*P*-value between two groups using the paired t-test. [b]*P*-value between two groups using the chi-square test.

Table 1 General and clinical data of repeated implantation failure patients (RIF) and pregnant controls.

	RIF group (n=40)	Control group (n=80)
Age (year) [a]	32.60±3.90	32.60±3.84
Infertility years (year) [a]	4.32±2.49	3.88±2.84
Infertility type (n)[b]		
Oviduct factors	20	33
Ovulation disorders	7	17
Oarium factors	3	9
Pelvic cavity factors	3	6
Male factors	3	7
Unknown causes	4	8
Endometrium thickness of transplant day (mm)[a]	11.00±3.66	12.17±1.04
High-quality rate of transplant embryo (%)[b]	87.75 (222/253)	90.91 (140/154)
BMI (kg/m2) [a]	21.07±2.85	21.26±2.55
AMH (ng/mL) [a]	3.59±2.55	3.24±2.13
D-dimer (mg/L) [a]	269.24±151.46	272.64±171.38
Fibrinogen (G/L) [a]	2.49±0.65	2.54±0.72
Basal hormone level		
Follicle-stimulating hormone (IU/L) [a]	5.36±2.37	5.26±2.13
Estradiol (pg/mL) [a]	31.42±12.08	34.19±21.19
Progesterone (ng/mL) [a]	0.63±0.29	0.65±0.52
Prolactin (ng/mL) [a]	14.18±6.16	13.87±6.46
Luteinizing hormone (IU/L) [a]	3.19±1.82	2.93±2.15
Testosterone (ng/mL) [a]	0.53±0.43	0.46±0.26
Transformation day hormone level		
Estradiol (pg/mL) [a]	559.87±269.49	547.29±367.50
Progesterone (ng/mL) [a]	0.47±0.27	0.50±0.33

All data are presented asthe mean ± SD. BMI: body mass index; AMH: anti-Mullerian hormone. [a]*P*-value between two groups using the paired t-test. [b]*P*-value between two groups using the chi-square test.

Table 2(on next page)

Differentially expressed proteins and their expression levels quantified by iTRAQ-2DLC-MS/MS.

Table 2 Differentially expressed proteins and their expression levels quantified by iTRAQ-2DLC-MS/MS.

Protein ID	Alternative name	Protein name	iTRAQ ratio
Increased in RIF/Controls			
Q9NYZ3	GTSE1	G2 and S phase-expressed protein 1	3.42
Q9UN19	DAPP1	Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide	3.38
Q5W111	SPRY7	SPRY domain-containing protein 7	3.11
P50225	ST1A1	Sulfotransferase 1A1	3.06
Q86UB9	TM135	Transmembrane protein 135	2.59
P05230	FGF1	Fibroblast growth factor 1	2.56
Q8NFU3	TSTD1	Thiosulfate:glutathionesulfurtransferase	2.49
Q8NDA2	HMCN2	Hemicentin-2	2.49
Q9H477	RBSK	Ribokinase	2.40
P15169	CBPN	Carboxypeptidase N catalytic chain	2.37
P02763	A1AG1	Alpha-1-acid glycoprotein 1	2.15
Q86VY4	TSYL5	Testis-specific Y-encoded-like protein 5	2.03
Q9UKJ8	ADA21	Disintegrin and metalloproteinase domain-containing protein 21	1.98
P02794	FRIH	Ferritin heavy chain	1.91
Q96RG2	PASK	PAS domain-containing serine/threonine-protein kinase	1.89
P08294	SODE	Extracellular superoxide dismutase [Cu-Zn]	1.88
P08582	TRFM	Melanotransferrin	1.87
Q9P2H3	IFT80	Intraflagellar transport protein 80 homolog	1.82
A0A0B4J1U7	1U7|HV601	Immunoglobulin heavy variable 6-1	1.81
P02765	FETUA	Alpha-2-HS-glycoprotein	1.78
Q9HCJ0	TNR6C	Trinucleotide repeat-containing gene 6C protein	1.78
P01008	ANT3	Antithrombin-III	1.77
Q14353	GAMT	Guanidinoacetate N-methyltransferase	1.77
P19652	A1AG2	Alpha-1-acid glycoprotein 2	1.77
Q86X19	TMM17	Transmembrane protein 17	1.75
O76041	NEBL	Nebulette	1.74
Q03167	TGBR3	Transforming growth factor beta receptor type 3	1.70
Q96EX3	WDR34	WD repeat-containing protein 34	1.67
P02792	FRIL	Ferritin light chain	1.66
Q9NWK9	BCD1	Box C/D snoRNA protein 1	1.64
P15559	NQO1	NAD(P)H dehydrogenase [quinone] 1	1.64

Protein ID	Alternative name	Protein name	iTRAQ ratio
Q96C11	FGGY	FGGY carbohydrate kinase domain-containing protein	1.64
Q99598	TSNAX	Translin-associated protein X	1.63
Q9NZM6	PK2L2	Polycystic kidney disease 2-like 2 protein	1.62
P08185	CBG	Corticosteroid-binding globulin	1.62
Q9BXN1	ASPN	Asporin	1.61
Q15063	POSTN	Periostin	1.61
Q9NVP4	DZAN1	Double zinc ribbon and ankyrin repeat-containing protein 1	1.60
Q14651	PLSI	Plastin-1	1.57
P02774	VTDB	Vitamin D-binding protein	1.57
P05543	THBG	Thyroxine-binding globulin	1.57
P25311	ZA2G	Zinc-alpha-2-glycoprotein	1.56
Q9NZJ9	NUDT4	Diphosphoinositol polyphosphate phosphohydrolase 2	1.55
Q9H649	NSUN3	tRNA (cytosine(34)-C(5))-methyltransferase, mitochondrial	1.55
P80108	PHLD	Phosphatidylinositol-glycan-specific phospholipase D	1.55
Q96AB6	NTAN1	Protein N-terminal asparagine amidohydrolase	1.54
Q9H9L4	KANL2	KAT8 regulatory NSL complex subunit 2	1.54
P02647	APOA1	Apolipoprotein A-I	1.53
P48509	CD151	CD151 antigen	1.53
Q9UBW7	ZMYM2	Zinc finger MYM-type protein 2	1.52
Q96LD8	SENP8	Sentrin-specific protease 8	1.52
P35237	SPB6	Serpin B6	1.52
Q5HYK9	ZN667	Zinc finger protein 667	1.51
Q99735	MGST2	Microsomal glutathione S-transferase 2	1.51
Q9NQG6	MID51	Mitochondrial dynamics protein MID51	1.50
Decreased in RIF/Controls			
Q9H9C1	SPE39	Spermatogenesis-defective protein 39 homolog	0.27
O43314	VIP2	Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase 2	0.36
P28908	TNR8	Tumor necrosis factor receptor superfamily member 8	0.40
Q96RD9	FCRL5	Fc receptor-like protein 5	0.44
P62805	H4	Histone H4	0.47
Q9P107	GMIP	GEM-interacting protein	0.48
Q96S82	UBL7	Ubiquitin-like protein 7	0.53

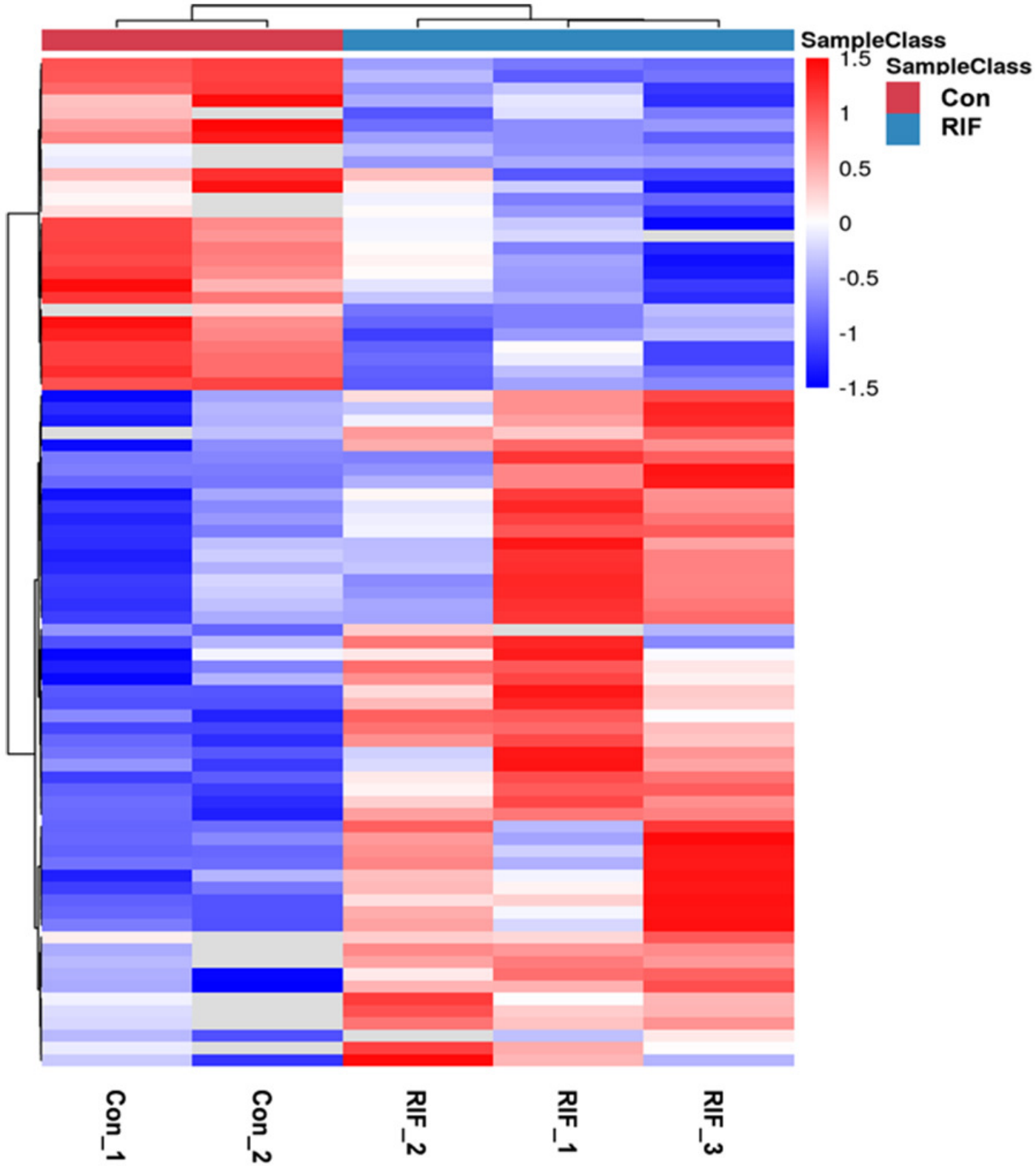
Protein ID	Alternative name	Protein name	iTRAQ ratio
P18124	RL7	60S ribosomal protein L7	0.53
P56202	CATW	Cathepsin W	0.53
Q6P179	ERAP2	Endoplasmic reticulum aminopeptidase 2	0.57
Q05086	UBE3A	Ubiquitin-protein ligase E3A	0.58
P43403	ZAP70	Tyrosine-protein kinase ZAP-70	0.59
P46781	RS9	40S ribosomal protein S9	0.59
Q8TAF3	WDR48	WD repeat-containing protein 48	0.60
Q8N4H5	TOM5	Mitochondrial import receptor subunit TOM5 homolog	0.60
P08729	K2C7	Keratin, type II cytoskeletal 7	0.61
Q07020	RL18	60S ribosomal protein L18	0.62
Q9BRX8	F213A	Redox-regulatory protein FAM213A	0.62
P07197	NFM	Neurofilament medium polypeptide	0.62
P15954	COX7C	Cytochrome c oxidase subunit 7C, mitochondrial	0.62
P01861	IGHG4	Immunoglobulin heavy constant gamma 4	0.64
P61313	RL15	60S ribosomal protein L15	0.64
Q96SI1	KCD15	BTB/POZ domain-containing protein KCTD15	0.65
P07196	NFL	Neurofilament light polypeptide	0.65
Q3SX64	OD3L2	Outer dense fiber protein 3-like protein 2	0.65
Q02543	RL18A	60S ribosomal protein L18a	0.65
Q8NGY6	OR6N2	Olfactory receptor 6N2	0.66

3

Figure 1

The hierarchical clustering for endometrium proteins between repeated implantation failure (RIF) and pregnant controls (Con) groups.

The red color showed the high expression, and the blue color represented the down expression. The color from red to blue represented the ratio from large to small.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SampleClass
Con
RIF
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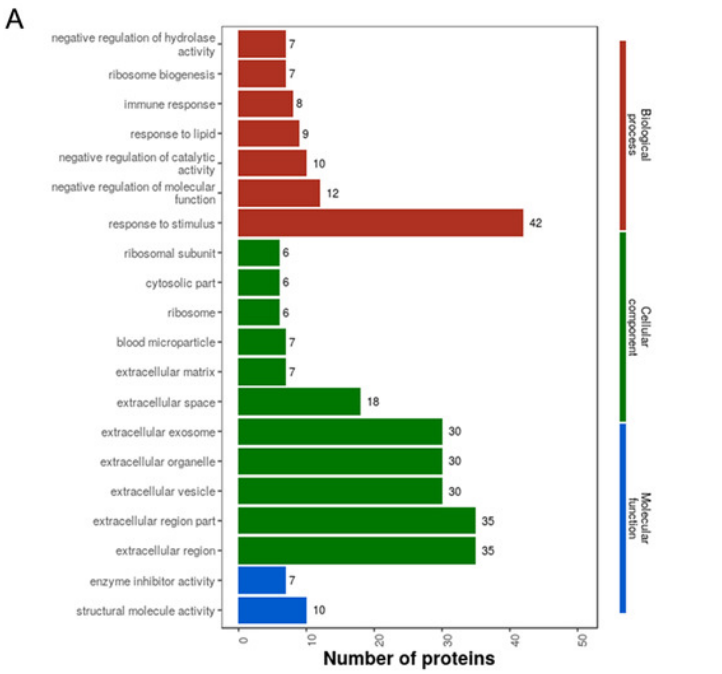
1
0.5
0
-0.5
-1
-1.5
Con_1
Con_2
RIF_2
RIF_1
RIF_3

Figure 2

GO analysis and String analysis of the set of endometrium proteins biomarker candidates for repeated implantation failure.

(A) GO analysis; (B) The enrichment analysis of GO terms; (C) Molecular function of GO terms; (D) String analysis.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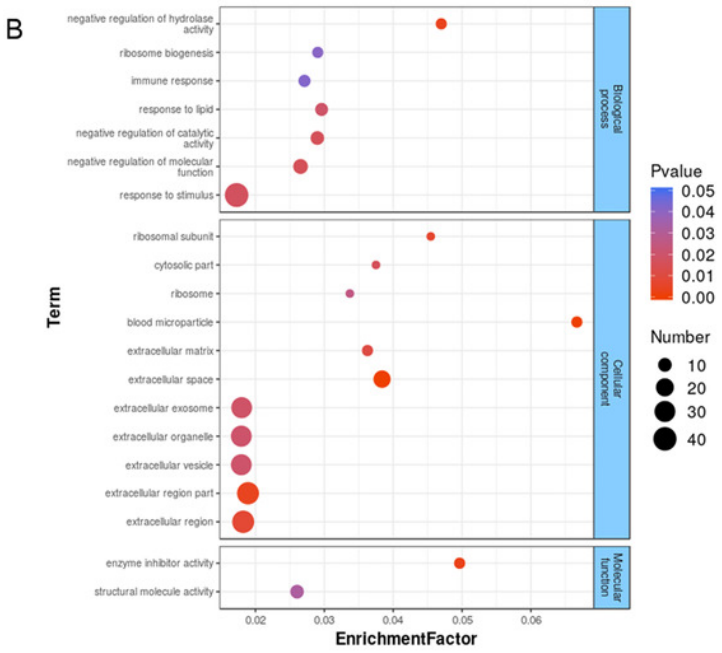
negative regulation of hydrolase activity
ribosome biogenesis
immune response
response to lipid
negative regulation of catalytic activity
negative regulation of molecular function
response to stimulus
ribosomal subunit
cytosolic part
ribosome
blood microparticle
extracellular matrix
extracellular space
extracellular exosome
extracellular organelle
extracellular vesicle
extracellular region part
extracellular region
enzyme inhibitor activity
structural molecule activity
7
7
8
9
10
12
42
6
6
6
7
7
18
30
30
30
35
35
7
10
0
10
20
30
40
50
Number of proteins
Biological process
Cellular component
Molecular function


B
negative regulation of hydrolase activity
ribosome biogenesis
immune response
response to lipid
negative regulation of catalytic activity
negative regulation of molecular function
response to stimulus
ribosomal subunit
cytosolic part
ribosome
blood microparticle
extracellular matrix
extracellular space
extracellular exosome
extracellular organelle
extracellular vesicle
extracellular region part
extracellular region
enzyme inhibitor activity
structural molecule activity
Term
0.02
0.03
0.04
0.05
0.06
EnrichmentFactor
Biological process
Cellular component
Molecular function
Pvalue
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0.04
0.03
0.02
0.01
0.00
Number
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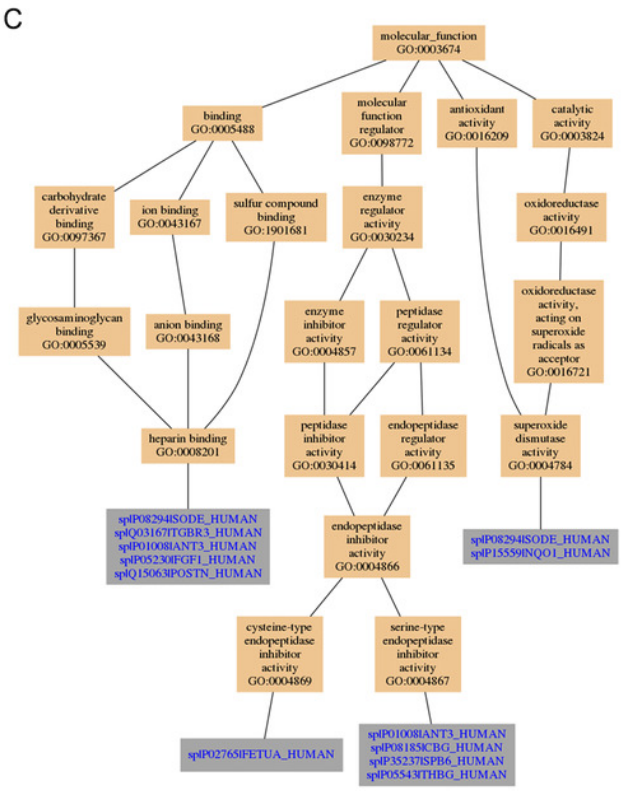
20
30
40


C
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binding GO:0005488
molecular function regulator GO:0098772
antioxidant activity GO:0016209
catalytic activity GO:0003824
carbohydrate derivative binding GO:0097367
ion binding GO:0043167
sulfur compound binding GO:1901681
enzyme regulator activity GO:0030234
oxidoreductase activity GO:0016491
glycosaminoglycan binding GO:0005539
anion binding GO:0043168
enzyme inhibitor activity GO:0004857
peptidase regulator activity GO:0061134
oxidoreductase activity, acting on superoxide radicals as acceptor GO:0016721
heparin binding GO:0008201
peptidase inhibitor activity GO:0030414
endopeptidase regulator activity GO:0061135
superoxide dismutase activity GO:0004784
splP08294|SODE_HUMAN
splQ03167|TGBR3_HUMAN
splP01008|ANT3_HUMAN
splP05230|FGF1_HUMAN
splQ15063|POSTN_HUMAN
endopeptidase inhibitor activity GO:0004866
splP08294|SODE_HUMAN
splP15559|NQO1_HUMAN
cysteine-type endopeptidase inhibitor activity GO:0004869
serine-type endopeptidase inhibitor activity GO:0004867
splP02765|FETUA_HUMAN
splP01008|ANT3_HUMAN
splP08185|CBG_HUMAN
splP35237|SPB6_HUMAN
splP05543|THBG_HUMAN

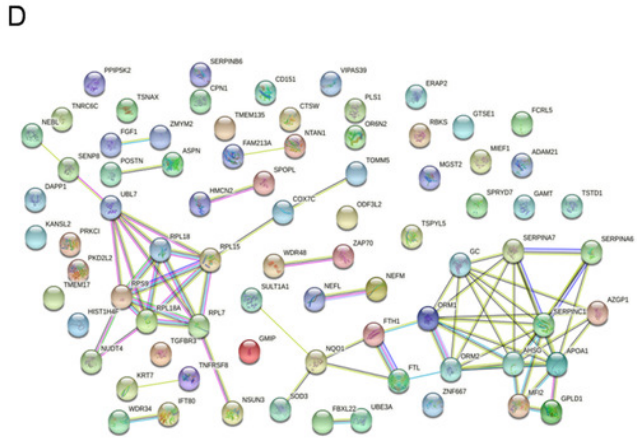
D

Figure 3

KEGG analysis and EggNOG analysis of the set of endometrium proteins biomarker candidates for repeated implantation failure.

(A) KEGG analysis; (B) The enrichment analysis of KEGG pathways; (C) EggNOG analysis.

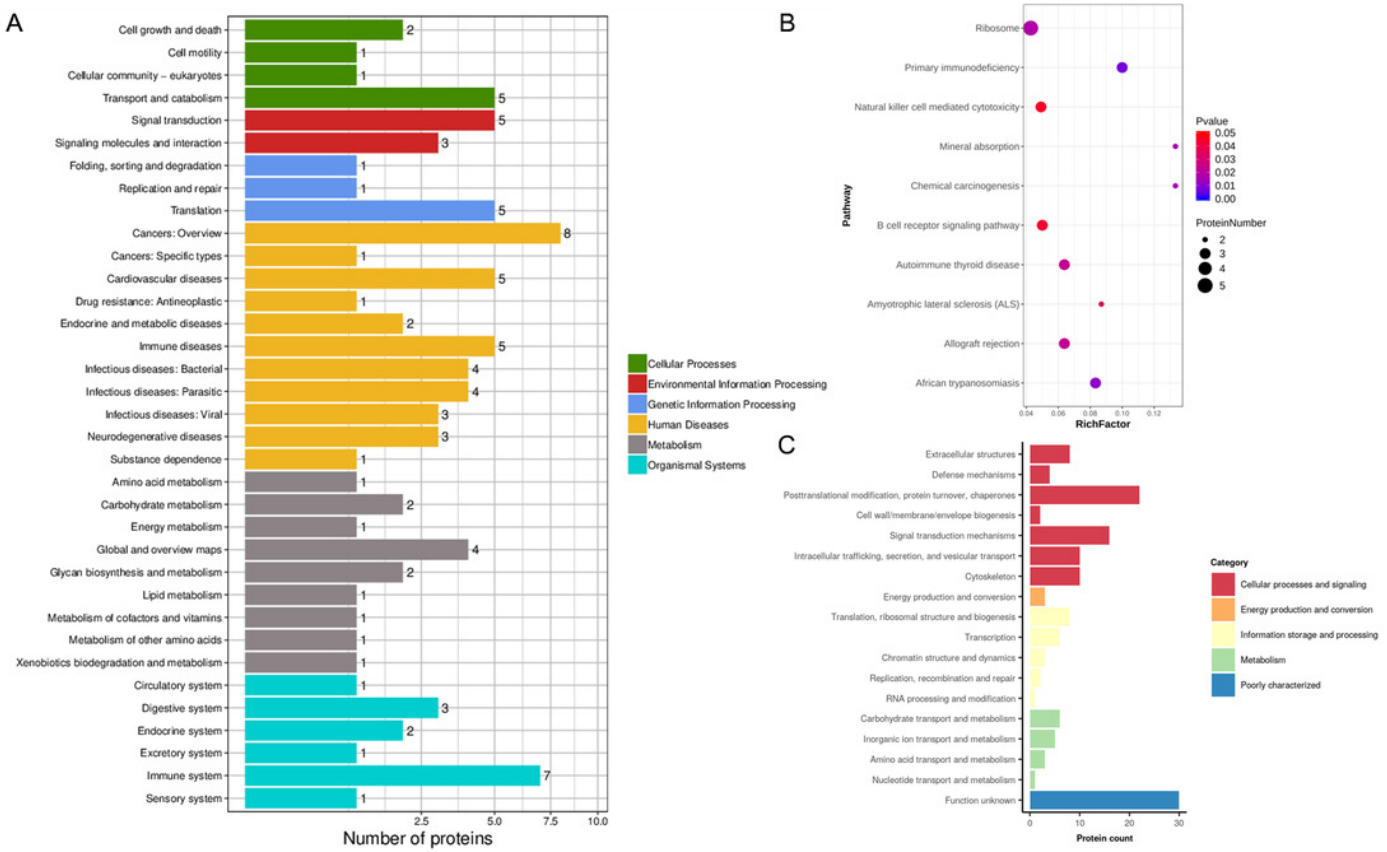

Figure 4

Westernblot results of candidate proteins.

GAPDH as internal reference.