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

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## Abstract

Inadequate endometrial receptivity may be responsible for the low implantation rate of transferred embryos in *in vitro* fertilization (IVF) treatments. Patients with repeated implantation failure (RIF) impact the clinical pregnancy rate for IVF. We collected endometrial tissue during the implantation window of hysteroscopy biopsies from September 2016 to December 2019 and clinical data were collected simultaneously. Patients were divided into RIF and pregnant controls group according to pregnancy outcomes. A total of 82 differentially expressed endometrial 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 the 2D LC MS/MS technique in the RIF group. String analysis found interactions between these proteins which assembled in two bunches: ribosomal proteins and blood homeostasis proteins. The most significant enriched Gene Ontology terms were negative regulation of hydrolase activity, blood microparticle, and enzyme inhibitor activity. Our results emphasized the corticosteroid-binding globulin and fetuin-A as the specific proteins of endometrial receptivity by Western-blot. Our study provided experimental data to establish the objective indicator of endometrial receptivity, and also provided new insight into the pathogenesis of RIF.

## Introduction

According to the World Health Organization, infertility is most prevalent in South Asia, Sub-Saharan Africa, North Africa/Middle East, Central/Eastern Europe, and Central Asia (Mascarenhas et al., 2012). There is a 25% infertility rate among couples of childbearing age in

39  China (Zhou et al., 2018). Assisted reproductive technology (ART) is currently the most
40  effective method to assist infertile patients (Kushnir et al., 2017).
41       In vitro fertilization and embryo transfer (IVF-ET) are the most common methods of ART.
42  Despite the success of these methods, some patients still have difficulty becoming pregnant even
43  after multiple transplantations (2-6 times) of high-quality embryos. These patients are classified
44  as repeated implantation failure (RIF) patients (Margalioth et al., 2006) and impact the clinical
45  pregnancy rate of ART. Embryo quality, which can be evaluated using several criteria, and
46  endometrial receptivity are the two key factors for successful implantation. Endometrial
47  receptivity is defined as the period during which the endometrial epithelium acquires a
48  functional, but transient, ovarian steroid-dependent status that supports blastocyst acceptance and
49  implantation. This period is called the window of implantation (WOI). Approximately 25.9% of
50  IVF-ET cases have a displaced WOI (Ruiz-Alonso et al., 2013), and the lack of synchronization
51  between the embryo and endometrial receptivity may be one of the causes of RIF. The standard
52  for evaluating endometrial receptivity is the pinopodes in endometrial histology but these can
53  change depending on the sample used and the period in the menstrual cycle (Acosta et al., 2000).
54  Ultrasound examination is a widely used, noninvasive and inexpensive test, and includes
55  endometrial thickness, endometrial type, endometrial volume, and uterine artery and sub-
56  endometrial blood flow. However, ultrasound examination has little ability to predict the
57  pregnancy rate with strong subjectivity. A number of studies have applied omics techniques to
58  analyze the human endometrium along different menstrual cycles, using biopsy or curettage
59  (Bissonnette et al., 2016; Chen et al., 2009; DeSouza et al., 2005; Parmar et al., 2009; Rai et al.,
60  2010; Ruiz-Alonso et al., 2012; Yap et al., 2011). Previous research (Bissonnette et al., 2016;
61  Chen et al., 2009; DeSouza et al., 2005; Parmar et al., 2009; Rai et al) used proteomic techniques
62  as 2D differentials in-gel electrophoresis (DIGE), Nanobore LC-MS/MS, and MALDI-TOF-TOF
63  to study the endometrium protein changes between the proliferative and secretory phase. Yap et
64  al. (2011) identified IL-11 regulated plasma membrane proteins ANXA2, and the lipid-raft
65  protein FLOT1 in human endometrial epithelial cells in vitro in the receptive phase.
66       We screened differential WOI endometrial proteins using iTRAQ labeling coupled with a
67  2D LC-MS/MS technique to find potential biomarkers for RIF patients. Our study provided
68  experimental data to establish an objective indicator of endometrial receptivity and a new way to
69  reveal the pathogenesis of RIF.
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## Materials & Methods

### Sample collection

73       This study was approved by the Ethics Committee of the Faculty of Medicine (Hangzhou
74  Women's Hospital, China) (Approval Number: 2016001-10). Written informed consent was
75  obtained from all subjects before endometrial collection.
76       The workflow of our study is shown in Fig. 1. Failure of three or more cycles in which
77  reasonably high-quality embryos were transferred was defined as RIF (Margalioth et al., 2006).
78  Grade I and grade II embryos were determined to be high-quality embryos (Gardner et al., 1998).

79   The blastocyst quality was determined according to the definitions by Gardner et al. (1998).
80   Blastocysts were considered high-quality if they had a grade 3 or 4 blastocoel, a grade A or B
81   inner cell mass, and a grade A or B trophectoderm on days 5 or 6. We collected data from fifty-
82   two RIF cases and 135 pregnancy cases undergoing IVF-ET treatment at our hospital between
83   September 2016 to December 2019. Data including age, follow-up outcomes, and clinical
84   examination findings were collected. The endometrium was collected at WOI by hysteroscopy
85   biopsy at LH+7. The endometrium was then washed with saline immediately and frozen in liquid
86   nitrogen and stored until protein extraction.

**87   Endometrium protein extraction**

88   We randomly selected 2 pregnancy cases and 3 RIF cases for endometrial protein extraction.
89   Protein extraction was performed using a lysis buffer after grinding the sample (100 mg) to
90   powder in liquid nitrogen. Phenylmethanesulfonyl fluoride (PMSF) was added to a final
91   concentration of 1mM and ethylene diamine tetraacetic acid (EDTA) was added to a final
92   concentration of 2mM, held for 5 min, and then dithiothreitol (DTT) was added to a final
93   concentration of 10mM. The sample was placed in an ice bath ultrasound for 5 min and the
94   lysate was centrifuged at 15,000 g for 20 min. The supernatant was precipitated 5 times with 1
95   mL acetone and incubated at 20 °C for 2 h and then centrifuged at 15,000 g at 4 °C for 20 min.
96   The precipitate was washed in chilled acetone, incubated at 20 °C for 30 min, and centrifuged
97   again at 15,000 g and 4 °C for 20 min. The washing was repeated twice, then air dried and the
98   precipitate was re-dissolved with the lysis buffer. The precipitate was centrifuged again at 15,000
99   g and 4 °C for 20 min after an ice bath ultrasound for 5 min. The supernatant was determined
100  using the Bradford Protein Assay Kit to detect the protein concentration.

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

102  A solution of 1:50 trypsin (Promega, USA)-to-protein mass was  prepared. Then, a total of
103  100 µg of protein from each group was digested with prepared solution at 37 °C for 16 h. The
104  peptides were reconstituted in 0.2M TEAB and processed according to the manufacturer's
105  protocol for 8-plex iTRAQ reagent (AB SCIEX, Framingham, MA, USA). Three biological
106  replicates of the RIF group were labeled with 113, 114, and 115 isobaric tags, respectively. The
107  peptides with two biological replicates from the pregnant group were labeled with 116 and 117
108  isobaric tags, respectively.
109  A high pH reversed-phase chromatography column (Phenomenex, Gemini-NX 3u C18110A,
110  150*2.00mm) was used for the first-dimensional fractionation procedure. We collected 16
111  fractions in all, and then dried them for next LC-MS analysis. The fractions were re-suspended in
112  2% acetonitrile containing 0.1% formic acid, and then loaded into a C18 trap column (Acclaim
113  PepMap 75µm × 150mm, C18, 3µm, 100A). Then, online chromatography separation was
114  performed on the nanoLC system (Dionex Ultimate 3000 RSLCnano) (Fan et al., 2019). The
115  trapping and desalting procedures were carried out at a flow rate of 3 µL/min for 5 min with 100%
116  solvent A (0.1% formic acid, 2% acet- onitrile and 98% water). The peptides were eluted using a
117  65 min gradient of buffer A (0.1% formic acid) to buffer B (80% ACN containing 0.1% formic
118  acid) at 300 nL/min on an analytical column (Acclaim PepMap 75 µm × 15 cm C18-CL, 3 µm

119    100 Å, Thermo160321). Q Exactive system (Thermo Scientific) fitted with a Nanospray ion
120    source was used to acquire tandem MS data. Specific steps are as follows: data were acquired
121    using an ion spray voltage of 2.2 kV. MS spectra across the scan range of 350–1800 m/z with a
122    70,000 resolution using maximum injection time (60 ms) per spectrum. Twenty of the most
123    intense precursors per MS cycle were selected for fragmentation and were detected with 100 ms
124    maximum injection time. Tandem mass spectra were recorded at a 17,500 resolution with the
125    rolling collision energy turned on and iTRAQ reagent collision energy adjustment turned on. The
126    lock mass option was enabled for more accurate measurements. Dynamic exclusion was set for
127    10 s.

128        Finally, the acquired MS/MS data were analyzed using IPeak and IQuant software as former
129    researchers (Wen et al., 2015; Wen et al., 2014; Fan et al., 2019). Only proteins identified at
130    global FDR ≤1% with ≥1 peptide were considered for further downstream analysis. A
131    differentially expressed protein was determine only if it is identified and quantified with at least
132    one significant peptide with the *P*<0.05 and fold change >1.5.

## Bioinformatics analysis

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

## Western-blot analysis

145        We lysed endometrial tissues with 200 μL of RIPA lysate (P0013B, Beyotime, Shanghai,
146    China) plus 1mM PMSF at 4 °C for 30 min, and then harvested the supernatant with
147    centrifugation at 11,000 g for 10 min. The harvested protein concentrations were measured with
148    a BCA quantitative kit (P0009, Beyotime). Samples were subjected to polyacrylamide gel
149    electrophoresis, and were transferred onto a PVDF membrane (IPVH00010, Millipore,
150    Massachusetts, USA). The membrane was blocked by 5% skimmed milk powder solution at
151    room temperature for 2 h and was incubated with primary antibodies, including antithrombin III
152    (rabbit monoclonal, ab126598, abcam, Cambridge, United Kingdom), cortisol binding globulin
153    (rabbit monoclonal, ab110648, abcam), fetuin-A (alpha-2-HS-glycoprotein, rabbit monoclonal,
154    ab137125, abcam), GAPDH (mouse monoclonal, 60004-1-Ig, proteintech, Beijing, China), and
155    alpha tubulin (rabbit polyclonal, 11224-1-AP, proteintech) at 4 °C overnight. Secondary
156    antibodies as goat anti-mouse IgG-HRP (BK0023, BEST, Xian, China) and goat anti-rabbit IgG-
157    HRP (BK0027, BEST) were then incubated with membrane at room temperature for 1.5 h. The

158   blots were visualized using the ECL Plus Luminous Kit (S17851, Yeasen, Shanghai, China). At
159   last, the results were measured with Image J software.

## Statistical analysis

161   Parametric data were tested using the chi-square test for the composition ratios and t-tests
162   for means of two groups. Nonparametric analysis was carried out using the Mann–Whitney U-
163   test. Parametric data were presented as mean ± SD while nonparametric data were presented as
164   median ± IQR, and $P < 0.05$ was considered to be statistically significant by the SPSS software,
165   version 16.0 (SPSS, Chicago, IL). Our clinical data was able to identify significant differences in
166   81.45% of RIF cases and controls at a statistical support level of α=0.05 with a d = 0.5 applying
167   a one tail model calculated by Gpower 3.0.5.

168

## Results

## Clinical data analysis

171   We recruited 52 RIF patients and 135 pregnant patients undergoing IVF-ET treatment
172   between September 2016 to December 2019. Data, including age, follow-up outcomes, and
173   clinical examination findings were collated into databases. After correcting for age, since age
174   may affect the pregnancy rate, a total of 40 subjects with RIF under 40 years old were paired
175   with 80 pregnant subjects by age, body mass index, and treatment time and then analyzed. There
176   were no significant differences between RIF patients and pregnant controls in general and
177   clinical data ($P$>0.05, Table 1).

## Endometrial proteomics results

179   We identified a total of 6,102 proteins through iTRAQ-2D LC-MS/MS from 113,384
180   spectra and 31,024 peptides, respectively (Supplementary Table 1). Among the 6,102 identified
181   proteins, 5,840 had GO annotations (95.71% of all proteins); 5,504 had KEGG annotations
182   (90.20% of all proteins); and 6,097 had EggNOG annotations (99.92% of all proteins).

183   We performed quality control on the quantitative results with volcano maps and the
184   distributions of coefficient of variation. We selected the fold change for 6,102 proteins and found
185   285 proteins with fold change in RIF cases/pregnant controls >1.5 or <0.67. And we only used
186   proteins with $P$ <0.05. Further screening revealed 82 differentially expressed proteins in RIF
187   patients compared with the pregnant controls, including 55 up-regulated proteins (>1.50-fold,
188   $P$<0.05) and 27 down-regulated proteins (<0.67-fold, $P$<0.05) (Table 2). The hierarchical
189   clustering provided a visualized mode to display the clustering patterns of the differentially
190   expressed proteins between the groups (Fig. 2).

## Bioinformatics analysis results

192   Gene Ontology analysis of differentially expressed proteins revealed that most of the
193   proteins were involved in the response to stimulus (42 proteins), extracellular region (35
194   proteins), and structural molecule activity (10 proteins) (Fig. 3A). The most significantly
195   enriched GOs were negative regulation of hydrolase activity, blood microparticle, and enzyme
196   inhibitor activity through hypergeometric testing (Figure 3B). Seven proteins (SPB6, APOA1,

197     GMIP, THBG, CBG, ANT3, and FETUA) were identified in the hydrolase activity term, seven
198     proteins (VTDB, IGHG4, APOA1, A1AG2, FETUA, ANT3, and A1AG1) were identified in the
199     blood microparticle term, and another seven proteins (ANT3, FETUA, CBG, ASPN, SPB6,
200     THBG, and APOA1) were identified in the enzyme inhibitor activity term. Among these
201     proteins, ANT3 and FETUA were identified in prior studies (Hannan et al., 2010; DeSouza et al.,
202     2005) which played an important molecular function in endopeptidase inhibitor activity by GO
203     analysis (Fig. 3C). String analysis found interactions between these proteins (Fig. 3D) which
204     assembled in two bunches: ribosomal proteins and blood homeostasis proteins.
205         In addition, the KEGG pathway mapping revealed the immune system (seven proteins),
206     transport and catabolism (five proteins), and translation (five proteins) pathways (Fig. 4A).
207     Enriched KEGG pathway analysis showed the ribosome and primary immunodeficiency
208     pathways as significant with $P<0.05$ (Fig. 4B). Finally, we used the EggNOG database to
209     determine that the differential proteins associated with RIF are mostly clustered in classifications
210     including posttranslational modification, protein turnover, chaperones (22 proteins), translation,
211     ribosomal structure and biogenesis (eight proteins), and carbohydrate transport and metabolism
212     (six proteins) (Fig. 4C).

213 **Western-blot results**

214         We verified endometrial antihrombin-III (ANT3, P01008), corticosteroid-binding globulin
215     (CBG, P08185), and fetuin-A (FETUA, P02765) levels using the Western-blot. We found
216     significantly higher levels of CBG and fetuin-A in RIF patients (Fig. 5). A significant difference
217     in CBG and fetuin-A was found in RIF patients using grayscale detection by Image J (1.39 fold,
218     $P=0.003$; 1.47 fold, $P=0.002$; respectively).

219

220 **Discussion**

221         Assisted reproductive technology has made rapid progress over the last 40 years. However,
222     the clinical pregnancy rate still only ranges between 33.8 and 42.7% (European IVF-Monitoring
223     Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE)
224     et al., 2016; Sunderam et al., 2019). After the implementation of blastocyst transplantation, the
225     clinical pregnancy rate increased to 60.4% (Ozgur et al., 2018). RIF is the key factor affecting
226     the pregnancy rate in IVF. Embryo quality, uterine factors (uterine cavity lesions, adenomyosis,
227     endometrial receptivity, etc.), immune factors (embryo immunity, maternal immunity), and a
228     multifactor effect could all lead to RIF but inadequate endometrial receptivity is the major cause
229     of decreased pregnancy success in RIF patients. We collected the endometrium of IVF-ET
230     patients during WOI and divided the samples into the RIF group and pregnant control group
231     according to pregnancy outcomes. Specific proteins related to endometrial receptivity were
232     screened using iTRAQ-2D LC-MS/MS.
233         Through iTRAQ-2D LC-MS/MS and bioinformatics analysis, 82 differential proteins were
234     obtained in the endometrium of RIF patients during the WOI, of which 55 were higher (> 1.50
235     times, $P< 0.05$) in RIF patients and 27 were lower (< 0.67 times, $P< 0.05$) in RIF patients. The
236     differential proteins obtained in this study have also been identified in the early proteomic

237  studies. Hannan et al. obtained seven differential proteins of the uterine lavage fluid of
238  pregnant/non-pregnant patients in the WOI by 2D-DiGE. The antithrombin III (ANT3, P01008)
239  was significantly increased in non-pregnant patients by immunohistochemistry (Hannan et al.,
240  2010). We found that the expression of ANT3 was 1.77 times as much as that in the pregnant
241  group, which was similar to previous results, indicating the reliability of iTRAQ-2D LC-MS/MS.
242  The results for ANT3, alpha-1-acid glycoprotein 1 (P02763), vitamin D-binding protein
243  (P02774), and FETUA (P02765) were also consistent with previous studies on the menstrual
244  phase (DeSouza et al., 2005). Ribosomal proteins and apolipoproteins have also been identified
245  in ours and other previous studies (DeSouza et al., 2005; Domínguez et al., 2009; Pérez-Debén et
246  al., 2019).

247      The differentially expressed proteins were related to the immune system and primary
248  immunodeficiency revealing by KEGG analysis and KEGG enrichment analysis (Fig. 3A, 3B).
249  Therefore, the change of immune response in RIF patients is self-evident. It has been shown that
250  the abnormal and functional defects of immune cells and molecules in endometrium during
251  implantation can lead to pregnancy failure (Liu et al., 2016). Therefore, we suspect that the
252  change of the endometrial immune microenvironment may lead to RIF, and may lead to a better
253  clinical treatment for RIF patients.

254      Our study revealed that most of the differentially expressed proteins were annotated with
255  "posttranslational modification, protein turnover, chaperones" and "translation, ribosomal
256  structure and biogenesis function" based on the EggNOG database, except for those with
257  "function unknown". So, we have reason to believe that modification after translation, synthesis
258  and degradation, folding, maintenance, intracellular transport, and mRNA translation might be
259  the key functions changed in embryo implantation. The results of enriched KEGG and String
260  analysis in our study also confirmed that translation was impacted.

261      The proteins in the most significantly enriched GOs contained ANT3 and FETUA, which
262  was consistent with results from previous studies (DeSouza et al., 2005; Hannan et al., 2010).
263  ANT3 was also related to the immune system as highlighted in the KEGG analysis. These
264  proteins were selected as candidates for validation. Protein was also selected based on a review
265  of the literature as CBG. Misao et al. (1995) suggested that the decrease of progesterone level in
266  the blood can lead to an increased CBG expression level in the endometrium. Low progesterone
267  levels may lead to higher miscarriage rates and lower live birth rates in frozen embryo transfer
268  patients (Gaggiotti-Marre et al. 2019).

269      Antithrombin-III is a representative protein of the prethrombotic state, which is encoded by
270  ANT3. The prethrombotic state is thought to be a major cause of RIF (Qublan et al., 2006).  Our
271  proteomics results corroborated those of Hannan et al. (2010), which revealed up-regulated
272  levels of antithrombin III in non-pregnant patients (Hannan et al., 2010). However, antithrombin
273  III showed no significant difference in RIF patients and pregnant controls. These differences may
274  be due to the differences in the validation tests used. We used the quantitative Western-blot for
275  validation while Hannan et al. used immunohistochemical localization. Therefore, antithrombin
276  III may not change in RIF patients.

277       Corticosteroid-binding globulin was a multifaceted component in cortisol delivery, also in
278    acute and chronic inflammation, and metabolism and neurocognitive function (Meyer et al.,
279    2016). The increased CBG level during pregnancy was important at the materno-fetal interface
280    (Lei et al., 2015). We found significantly higher levels of CBG in RIF patients (Fig. 4). Misao et
281    al. (1995) suggested that the decrease of progesterone levels in the blood may increase CBG
282    expression in the endometrium. However, we found a lower level of serum progesterone in RIF
283    patients, but the difference was not significant (Table 1) and a larger sample of research may be
284    needed. The endometrial CBG content is thought to originate in the plasma (Kreitmann et al.,
285    1978), thus, RIF patients may have a positive outcome with supplemental progesterone
286    administration.

287       Fetuin-A is defined as the inhibitor of ectopic calcification in circulation, which also takes
288    part in multiple metabolic pathways such as insulin resistance, vascular calcification, and
289    inflammation (Bilgir et al., 2010; Ishibashi et al., 2010; Wang et al., 2012). Fetuin-A is also
290    popular in studies on adverse pregnancy outcomes like pre-eclampsia (Sanhal et al., 2016) and
291    gestational diabetes mellitus (Kansu-Celik et al., 2019). A previous study on pre-eclampsia
292    concluded that fetuin-A may decrease trophoblast viability and invasion caused by the inhibition
293    of receptor tyrosine kinase activity (Gomez et al., 2012). Our study supported the results that
294    showed that elevated fetuin-A may lead to failed implantation and cause an adverse pregnancy
295    outcome. Meanwhile, Ozgu-Erdinc et al. (2020) demonstrated that serum fetuin-A level were
296    also increased in implantation failure patients in IVF cycles. Additional studies on the regulation
297    of the level of fetuin-A as a treatment strategy may improve implantation success.

298

## Conclusions

300       We screened the endometrial proteomics of RIF patients using iTRAQ-2D LC-MS/MS at
301    WOI, revealing that the endometrial immune microenvironment may lead to RIF. Our validated
302    results confirmed CBG and fetuin-A as the specific protein for RIF patients. Our results provide
303    experimental data to establish the objective indicator of endometrial receptivity and give a new
304    insight into the pathogenesis of RIF.

305

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

All data are presented as the mean ± SD. BMI: body mass index; AMH: anti-Mullerian hormone. [a]P-value between two groups using the t-test, [b]P-value between two groups using the chi-square test, [c]P-value between two groups using the Mann-Whitney U-test.

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

2 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] | 9.71±1.77 | 10.52±2.27 |
| 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) [c] | 220.00±202.50 | 210.00±230.00 |
| Fibrinogen (G/L) [a] | 2.49±0.65 | 2.54±0.72 |
| Basal hormone level | | |
|   Follicle-stimulating hormone (IU/L) [a] | 5.30±2.37 | 5.26±2.13 |
|   Estradiol (pg/mL) [c] | 27.00±17.30 | 25.00±22.50 |
|   Progesterone (ng/mL) [a] | 0.62±0.29 | 0.65±0.52 |
|   Prolactin (ng/mL) [a] | 14.02±6.18 | 13.87±6.46 |
|   Luteinizing hormone (IU/L) [a] | 3.14±1.83 | 2.93±2.15 |
|   Testosterone (ng/mL) [a] | 0.53±0.43 | 0.46±0.26 |
| Transformation day hormone level | | |
|   Estradiol (pg/mL) [a] | 554.33±268.31 | 585.78±398.97 |
|   Progesterone (ng/mL) [a] | 0.48±0.27 | 0.52±0.34 |

3 All data are presented as the mean ± SD. BMI: body mass index; AMH: anti-Mullerian hormone. [a]*P*-value

4 between two groups using the t-test, [b]*P*-value between two groups using the chi-square test, [c]*P*-value

5 between two groups using the Mann-Whitney U-test.

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

1  **Table 2** Differentially expressed proteins and their expression levels quantified by iTRAQ-

2  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 |

# Figure 1

The workflow for endometrial biomarkers of repeated implantation failure (RIF) and pregnant controls (CON).

```
┌──────────────────────────┐         ┌──────────────────────────┐
│  Repeated implantation   │         │   Pregnant controls      │
│   failure patients       │         │        (CON)             │
│        (RIF)             │         │                          │
└──────────────────────────┘         └──────────────────────────┘
```

**Clinical data analysis**
(RIF, n=40; CON, n=80)

**iTRAQ-2D LC-MS/MS**
(RIF, n=3; CON, n=2)

Extraction and Quantitative of
Endometrium protein

Preconditioning and iTRAQ labeling
(113, 114, 115: RIF; 116, 117: CON;
including two biological replicates)

2DLC-MS/MS
(Q Exactive system, 113,384 spectra identified)

Protein identification and
quantification
(IPeak and IQuant software)

6,102 proteins identified

*P*-values < 0.05        fold change > 1.5

82 differentially expressed proteins
(55 up-regulated and 27 down-regulated)

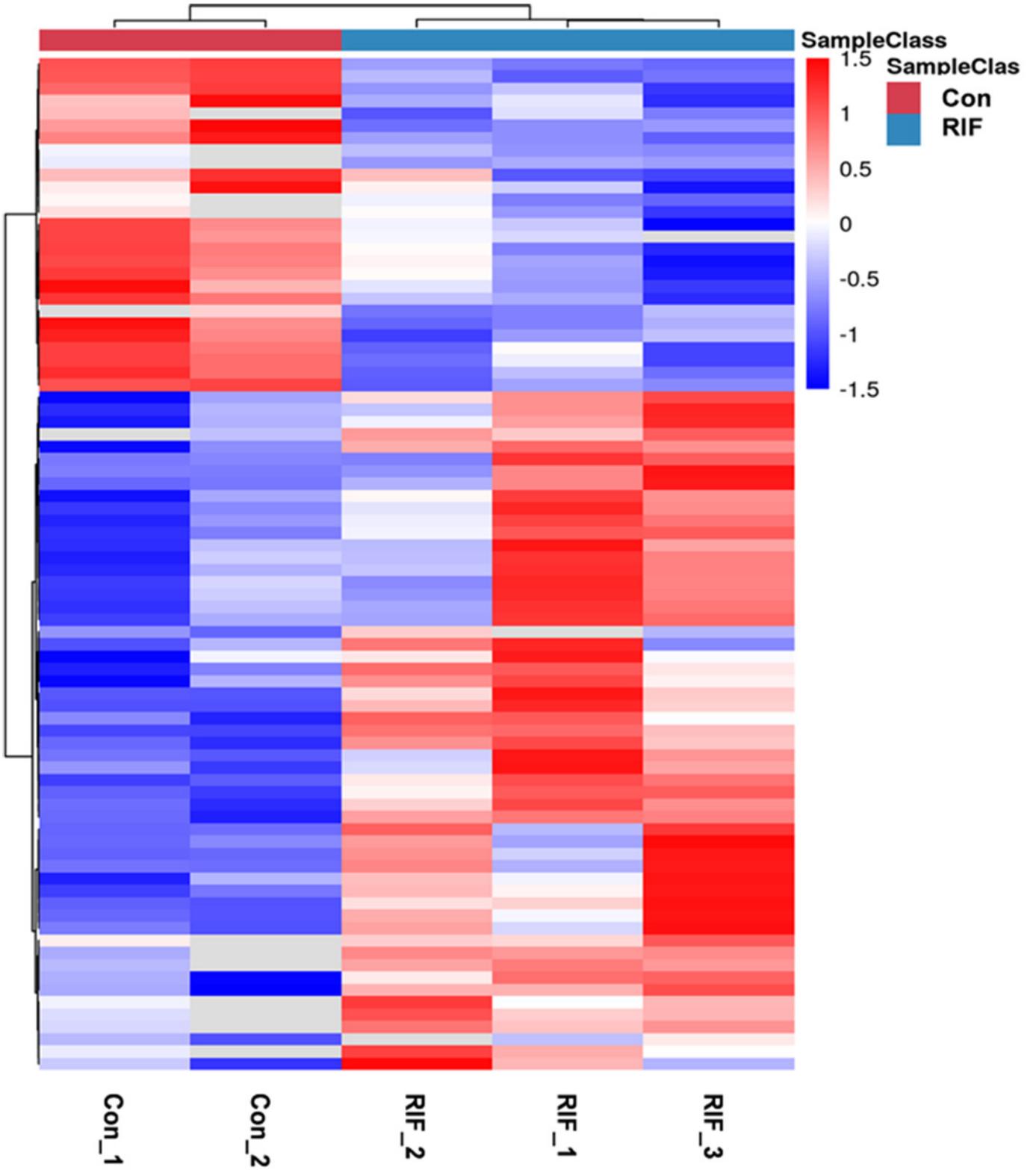
**Bioinformatics analysis**
GO classification and
hypergeometric test;
KEGG enrichment analysis;
STRING analysis;
EggNOG analysis.

literature review

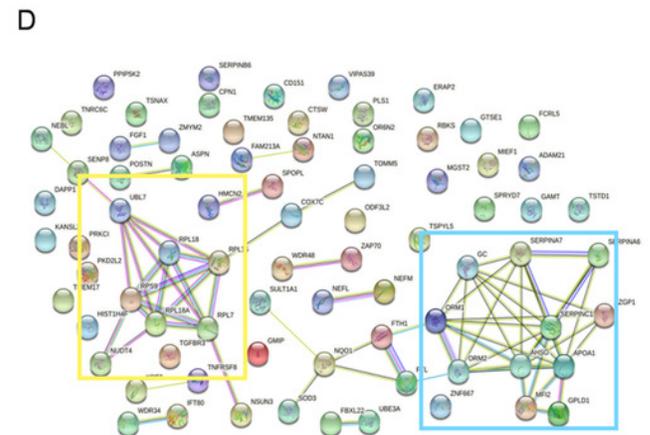
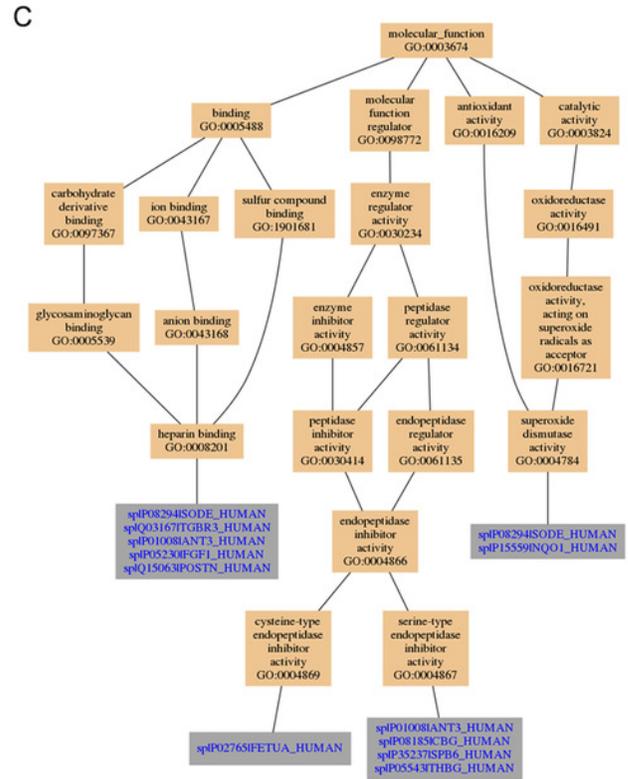
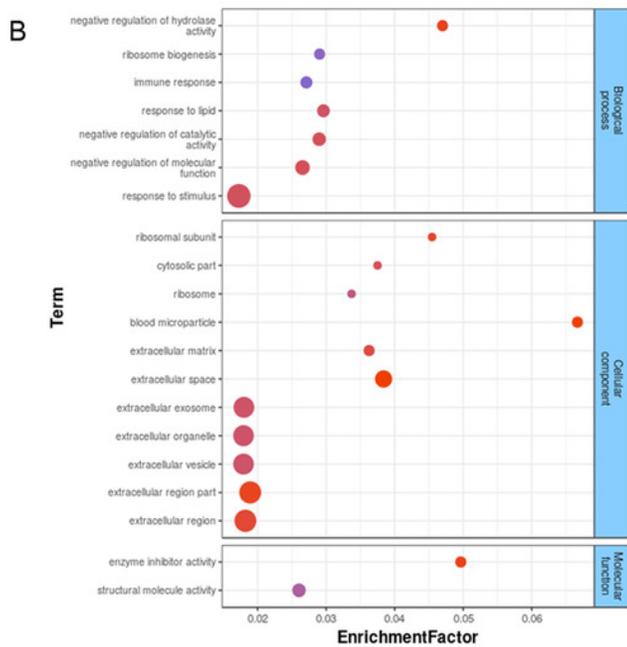
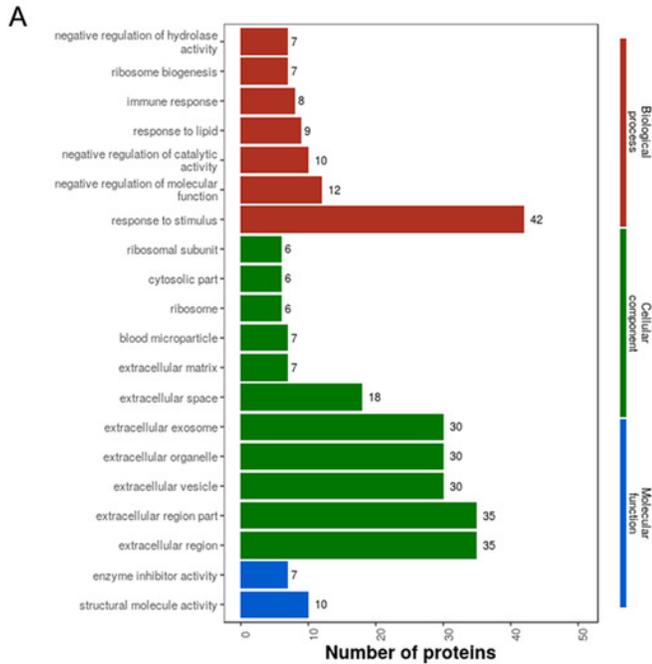
**Western-blot validation**
(ATIII , CBG, Fetuin-A; RIF, n=9; CON, n=9)

# Figure 2

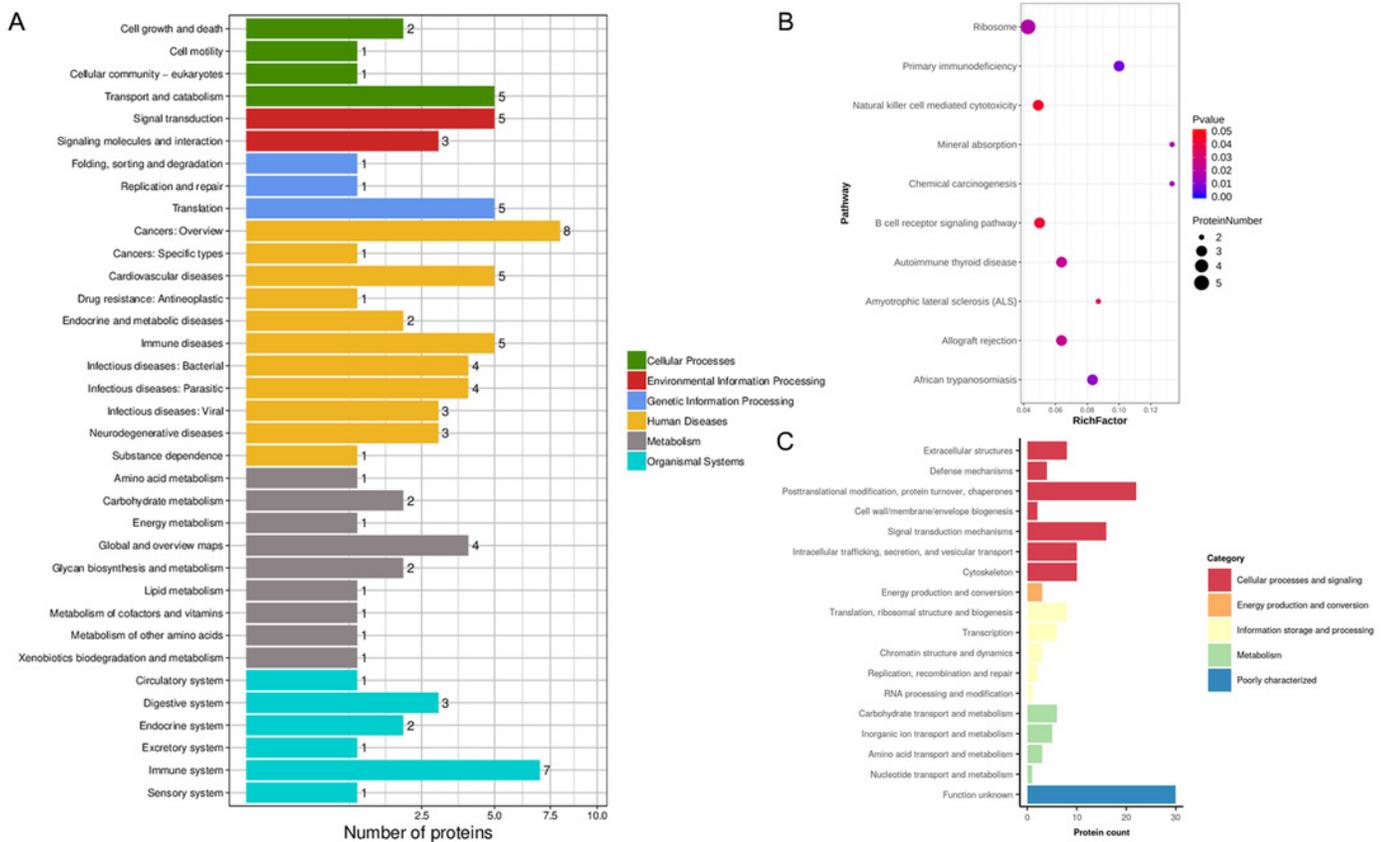
The hierarchical clustering for endometrium proteins between repeated implantation failure (RIF) and pregnant controls (Con) groups.

The red color showed the up-regulated expression, and the blue color represented the down-regulated expression. The color from red/blue to white represented the ratio from large to small.

# Figure 3

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

(A) GO analysis of 82 differentially expressed proteins revealed 7 significant GO terms in biological process, 11 significant GO terms in cellular component, and 2 significant GO terms in molecular function ($P < 0.05$). (B) The enrichment analysis revealed 20 significant GO terms by hypergeometric test ($P < 0.05$). GO terms with bigger enrichment factor indicate the greater degree of enrichment. (C) Molecular function of GO terms for proteins identified both in our and prior studies. (D) Network nodes represent proteins while edges represent protein-protein associations which were already known (light blue and purple) or predicted (other colors) by String analysis. Proteins enclosed in color-coded outlines are mainly involved in ribosomal proteins (yellow) and blood homeostasis proteins (blue).

# Figure 4

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

(A) KEGG pathway analysis of 82 differentially expressed proteins using KEGG database revealed 35 pathways. (B) The enrichment analysis of KEGG pathways revealed 10 significant pathways by hypergeometric test ($P < 0.05$). Pathways with bigger rich factor indicate the greater degree of enrichment. (C) EggNOG analysis predicted pairwise orthology and functional classification for 82 differentially expressed proteins.

# Figure 5

Proteins expression levels change between repeated implantation failure (RIF) and pregnant controls (CON).

(A) antithrombin III (ATIII) (52 KDa); (B) corticosteroid-binding globulin (CBG) (45 KDa); and (C) fetuin-A (39 KDa) were analyzed by western-blot in nine cases of pregnant controls (CON) and repeated implantation failure (RIF) patients. Beside each western-blot picture, grayscale analysis is represented where the intensity of each protein band is compared to a GAPDH/tubulin band. Grayscale analysis showed a similar tendency to the iTRAQ analysis, with a higher protein abundance of CBG in RIF cases (1.39 fold, *P*=0.003), also higher fetuin-A (1.47 fold, *P*=0.002).