RPA2 Impairs the Endometrial Decidualization in Repeated Implantation Failure Patients

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Research Article

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Abstract

**Background:** Insufficient endometrial receptivity contributes to repeated implantation failure (RIF), which is associated with aberrant proliferation and decidualization of human endometrial stromal cells.

**Methods:** In this study, we retrieved the expression profiles from GEO databases and filtered the differentially expressed genes between RIF and the fertile control group. Ultimately, RPA2 was confirmed as target gene. RPA2 expression in endometrial tissues of RIF patients, the control group and different phases was detected by RT-qPCR, immunohistochemistry and western blotting. The role of RPA2 in endometrial decidualization was performed by in vitro decidualization inducing by 8-Br-cAMP and MPA.

**Results:** RPA2 was significantly upregulated in the mid-secretory endometrium of patients with RIF. As a proliferation-related gene, RPA2 was obviously higher expressed in the proliferative phase than in the mid-secretory and decidualized tissues. Moreover, the downregulation of RPA2 was discovered during decidualization of HESC which revealed that the proliferation ability decreased with the decidualization of the endometrium.

**Conclusions:** Our finding indicated that upregulation of RPA2 disrupted the homeostasis between proliferation and decidualization of HESC and further reduced endometrial receptivity of RIF women.

Introduction

Repeated implantation failure (RIF) refers to a failure of achieving clinical pregnancy after implanting one or two good-quality embryos at least three times during in vitro fertilization-embryo transfer (IVF–ET) [1, 2]. It is one of the main causes of infertility and a severe and challenging problem in assisted reproductive technology (ART) field. Successful embryo implantation needs synchrony in the development of good endometrial receptivity and functional quality embryos [3]. Currently, endometrial dysfunction has been considered the decisive cause of RIF [4].

The human endometrial cycle is divided into two phases: the proliferative phase which is a period of vigorous growth of endometrial epithelial cells and mesenchymal cells, and the secretory phase, during which endometrium experiencing decidualization to be ready for implantation [5]. The establishment of the window of implantation (WOI) phase and successful clinical pregnant requires proper transition from the proliferative to secretory phase. The inappropriate transition between these two phases and decidualization deficiency were appeared in RIF patients [6, 7]. Decidualization is an essential and typical change in the endometrium during blastocyst implantation, including the proliferation, differentiation and decidualization of endometrial stromal cells. Appropriate proliferation of endometrial stromal cells is indispensable for decidualization, and attenuating proliferation restrains decidualization and further leads to blastocyst implantation failure [8]. For example, Notch 1 could regulate cell proliferation and facilitate successful decidualization of endometrial stromal cells, and Notch1 deficiency in mice resulted in fewer offspring[9]. The significantly lower PIBF1, IL6, and p-STAT3 expression notably inhibited the proliferation and decidualization of endometrial stromal cells[10]. Therefore, exploring new molecular and mechanism of affecting proliferation and decidualization contributes to understand the initiation and progression of RIF.
To elucidate how the endometrial gene expression profile differs between women with RIF and controls, we downloaded the mRNA expression profiles (GSE58144 and GSE111974) of endometrium tissues from the Gene Expression Omnibus (GEO) database. After screening and verifying, RPA2 was selected as hub target gene with significantly differential expression. RPA2 is a subunit of the Replication Protein A complex, which plays an important role in DNA replication, DNA repair and homologous recombination[11]. DNA damage-induced RPA2 hyperphosphorylation involved in cell-cycle arrest and further influenced the cells proliferation [12]. Nevertheless, it is unclear whether RPA2-mediated growth is responsible for proliferative-secretory phase transition, embryo implantation during decidualization and the pathogenesis of RIF. In this study, we compared the expression of RPA2 in mid-secretory endometrium between RIF patients and the healthy women at multiple levels. In addition, we examined the RPA2 expression in proliferative phase, secretory phase, decidual tissue, and endometrial stromal cells with induce decidualization. This study aimed to clarify the crucial role of RPA2 expression in the cause of RIF and the decidualization of endometrial stromal cells.

Materials And Methods

Microarray data acquisition

Two expression profiles (GSE58144 and GSE111974) were retrieved from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/), which is a free public repository and a recording platform used for searching gene expression datasets. GSE58144 is based on GPL15789 platform (A-UMCU-HS44K-2.0), containing 43 RIF and 72 normal endometrium specimens. GSE111974, the platform of which was the GPL17077 (Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray 039381 (Probe Name version)), included 24 tissues of RIF and 24 normal endometrium samples of fertile control patients. The basic information of these two profiles was shown in Table 1. The data analysis of was processed by bioconductor limma (versions 3.30.0) R package. The differentially expressed genes (DEGs) were acknowledged with p value <0.05 in GSE58144 and p value <0.05 in GSE111974.

GO term and KEGG pathway enrichment analysis

The Gene Ontology (GO, http://geneontology.org/) annotation was performed to evaluate the biological characteristics of DEGs, consisting of biological processes, cellular components, and molecular functions[13]. Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/) pathway enrichment analysis was conducted to find key pathways the DEGs participated in RIF [14]. The associated data was downloaded from the official websites and depicted using clusterProfiler(versions 3.18.0) package in R (R-3.6.0).

Construction of PPI network and identification of hub genes

The protein-protein interaction (PPI) network was conducted to visualized the interaction among the differentially expressed proteins based on the Search Tool for the Retrieval of Interacting Genes (STRING) database(http://string-db.org/, version 11.0) [15] using cytoscape software (version 3.7.0). Subsequently, the hub genes with degree > 10 were appraised bycytoHubba plugin in cytoscape. Also, MCODE module was used to find closely connected nodes in a complex network.

Endometrial tissues
This study was approved by the Medical Ethics Committee of Yantai Yuhuangding Hospital. All participants signed informed consent forms approved by the Institutional Review Board of Yantai Yuhuangding Hospital (reference number 2019-121) before performing any study-related procedure.

24 RIF patients who did not get pregnancy after at least three IVF–ET failure cycles and 24 control women who experienced IVF–ET cycle due to tubal obstruction without hydrosalpinx achieved a clinical pregnancy after their first or second embryo transfer were enrolled. The exclusion and inclusion criteria followed in our previous study [16]. The characteristics of these patients were listed in Table 2. Simultaneously, 20 infertility women with tubal factor and 10 healthy women with abortion were selected followed the above exclusion and inclusion criteria. These 20 infertility women were divided into two groups (n = 10 per group): proliferative phase and secretory phase. Endometrial tissue samples were obtained from all above enrolled patients. The characteristics of these 30 patients were shown in Table 3.

Cell culture and primary cells isolation

HESCs (human endometrial stromal cells), which were kind gifts from Professor Haibin Wang (School of Medicine, Xiamen University, Xiamen, China), were cultured in phenol red-free DMEM/F12 (Gibco BRL/Invitrogen, Carlsbad, CA, USA) supplied with 10% Charcoal Stripped Foetal Bovine Serum (Biological Industries, Israel).

Primary endometrial epithelial cells and stromal cells were isolated from the mid-secretory phase endometrial tissues. First, we minced the endometrial tissues with sterile ophthalmic scissors and then enzymatically digested with 0.15% (w/v) collagenase I (Sigma-Aldrich, St. Louis, Mo, USA) for 60 min at 37 °C. Next, the digested tissues were passed through a 100 μm-sieve to remove tissue blocks, and subsequently separated stromal cells from epithelial cells through a 40 μm filter.

In vitro decidualization

HESCs were treated with 0.5 mM 8-Br-cAMP (Sigma-Aldrich, St. Louis, Mo, USA) and 1μM methylprogesterone acetate (MPA, MedChemExpress LLC, New Jersey, USA) and assessed following a period of incubation (overnight, day 2, day 4, day 6 and day 8). Differentiation was evaluated by measuring two decidualization-specific markers (prolactin [PRL] and insulin-like growth factor-binding protein 1 [IGFBP1]) and by examining cell morphology.

RNA isolation and RT-qPCR

Total RNA was extracted from cells and tissue samples using Trizol reagent following manufacturer’s instructions (Shandong Sparkjade Biotechnology Co., Ltd., China). Purified 1 μg RNA was reverse-transcribed to generate cDNA using SPARKscript II RT Plus Kit (Shandong Sparkjade Biotechnology Co., Ltd., China). The expression of mRNAs in these individual samples was performed by Reverse transcription quantitative real-time PCR (RT-qPCR) reaction using SYBR Green qPCR Mix kit (Shandong Sparkjade Biotechnology Co., Ltd., China) following: 94°C for 2min, followed by 40 cycles of 95°C for 10s and 60°C for 30s. The primer sequences used of targeting genes were shown in Table 4.

Western blotting
Total protein was extracted from endometrial tissues or cells using RIPA buffer (Shandong Sparkjade Biotechnology Co., Ltd., China) containing protease inhibitors. Equal amounts of protein (30 mg) were separated using 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. After blocking with 5% skimmed milk for 1 hour at room temperature, the membranes were incubated with antibodies against RPA32/RPA2 (1:1000; 35869T; Cell Signaling Technology), GAPDH antibody (1:1000; D110016-0100; Sangon Biotech). Next day, membranes were incubated with a HRP Conjugated Goat anti-Rabbit IgG h+1 secondary antibody (1:5000; abs20040ss; absin). The bands were analyzed using the Image J software.

**Immunohistochemical staining**

The expression and localization of RPA2 was assessed by immunohistochemistry (IHC). All tissues were fixed with 4% paraformaldehyde and embedded in paraffin. 4-mm-thick tissue sections were got from the paraffin tissue block. First, the tissue sections were grilled in oven with 65 °C for 1 hour. Then slides were deparaffinized in fresh xylene for 20 min (Xylene I 10 min, Xylene II 10 min) and rehydrated through graded ethanol series (100% alcohol I 10 min, 100% alcohol II 10 min, 95% alcohol I 10 min, 95% alcohol II 10 min, 70%alcohol 10 min). After washing the slides with water three times, antigen retrieval was conducted in a microwave oven at 100°C for 20 min, with subsequent cooling to room temperature. Subsequently, the immunohistochemical sections were incubated with 3% H2O2 for 10 min to block endogenous peroxidases in a wet box. After blocking with 3%BSA, the slides were incubated overnight at 4 °C with RPA2 (1:200; 35869T; Cell Signaling Technology). Next day, the sections were washed with PBS and incubated with secondary antibody, then visualized with 3,3-diaminobenzidine (DAB) and hematoxylin (counterstain). Finally, all slides were dehydrated using a gradient grade ethanol and xylene series according to routine dehydration steps, and fixed with neutral resin. Normal serum was used instead of primary antibody as negative control.

**Statistical analysis**

The continuous variables were shown as means ± standard deviation. The RT-qPCR results were tested by the Student’s t-test or one-way ANOVA analysis. All statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). When p<0.05 was believed as statistically significant.

**Results**

**Microarray data information and identification of DEGs in RIF endometrial tissues**

The basic information of two GEO datasets (GSE111974 and GSE58144) was shown in Table 1. 1666 DEGs (678 upregulation and 988 downregulation) with p<0.05 were filtered out from GSE58144 using a limma (versions 3.30.0) R package (Figure 1A). Also, we screened out 4508 DEGs (1972 upregulation and 2536 downregulation) with p<0.01 from GSE111974 (Figure 1B) using the same data processing method. Subsequently, 162 overlapping downregulated DEGs (Figure 1C) and 74 overlapping upregulated DEGs (Figure 1D) were screened in the RIF group compared with the control group using Venn software online. The top 20 downregulated and upregulated DEGs in the integrated microarray analysis were mapped on a heat map using a RRA package (Figure 1E).
GO and KEGG enrichment analysis

To explore the biological functions and pathway of DEGs in RIF, GO annotation analysis were conducted using the DAVID online analysis tool. The biological process, cellular component and molecular function terms of upregulated DEGs were showed in Figure 2A. As shown in Figure 2B, KEGG pathway analysis revealed the upregulated DEGs in RIF were significantly enriched in ‘Apoptosis’, ‘NF-kappa B signaling pathway’, ‘AMPK signaling pathway’, and ‘Cell adhesion molecules’ et al. related signaling pathway. The downregulated DEGs was significantly enriched in DNA replication or repair related biological functions Figure 2C. As shown in Figure 2D, KEGG pathway analysis of the downregulated DEGs were conducted in RIF, of which the critical pathway were ‘DNA replication’, ‘DNA repair’, ‘Metabolic pathways’ and ‘Cell cycle’. Therefore, the DEGs were mainly associated with DNA replication or repair.

PPI network construction and hub gene validation

Based on the STRING database, a PPI network was established for further investigation of the interaction among the DEGs, which was visualized using the Cytoscape software. The PPI network consisted of 145 nodes and 223 edges, among which comprising 102 downregulated and 43 upregulated genes (Figure 3A). 4 downregulated genes with degree > 10 were identified as hub genes, which was RFC4, PCNA, PLK4 and RPA2, respectively. Subsequently, we explore the significant clusters in this PPI network, two clusters closely connected was discovered (Figure 3B, C). Interestingly, four target genes (RFC4, PCNA, PLK4 and RPA2) were all uncovered in the second cluster (Figure 3C), which demonstrated that these four genes not only had high degree, but also hold close relationship among them. Further, we investigated the biological functions and signaling pathways of these four hub genes participated in. As shown in the GO circle plot and KEGG circle plot of Figure 3D, E, they were mainly associated with DNA replication or repair and cell cycle.

Hub gene validation

Aimed to confirm the four candidate hub genes were dysregulated in RIF, we detected their expression in endometrial tissues of RIF women compared with the control group. The results illustrated that all the four target genes (RFC4, PCNA, PLK4 and RPA2) were not consistent with the predicted results, but the difference of RFC4 between two groups was not significant (Figure 4B). Owing to we found more fun of PCNA, we would report the related results of PCNA in the future study, but not in this research. PLK4 was reported as a key gene, regulatory factor, and drug target gene of RIF, which was downregulated in RIF [17]. In addition, the abnormal expression of PLK4 of human preimplantation embryo could lead to the tripolar mitosis and aneuploidy, which would result in IVF failure [18]. In addition, PLK4 was downregulated in the endometrium of different phase (proliferative phase, mid-secretory phase and decidual tissues), whereas there was no difference in RFC4 among three phases (Figure 4C, D). Therefore, RPA2 was selected as the key research object of this study.

RPA2 is increased in the endometrium of RIF patients

The above findings encouraged us to further explore the mission of RPA2 in the endometrium. Immunohistochemical staining revealed stronger staining of RPA2 in RIF endometrium than in healthy endometrium of mid-secretory phase, and RPA2 was expressed in both stromal cells and epithelial cells
Moreover, western blotting was applied to validate that RPA2 was adequate expression in the endometrium of RIF patients compared with the control group (Figure 5B), consistent with the qPCR results (Figure 5D). All the sections were in mid-secretory phase.

RPA2 is deficient in the decidual tissues and is inhibited by 8-Br-cAM Pang MPA

Due to decidualization of stromal cells is a key step for the uterus to receive embryos, and decidualization dysregulation may lead to implantation failure, thence we investigate the role of RPA2 in decidualization of stromal cells. To explore the pathophysiological significance of RPA2 during early pregnancy, we detected the expression pattern of RPA2 expression using immunohistochemistry and qPCR. The results suggested that RPA2 protein abundance gradually decreased during the menstrual cycle (Figure 6A), and its tendency at protein level was consistent with mRNA (Figure 6B), which showed RPA2 was lowest in the decidual tissues. Moreover, higher expression of RPA2 was observed in endometrial epithelial cells than stromal cells, and the differential expression of RPA2 in these two type cells was further tested and verified in primary endometrial epithelial cells and stromal cells by qPCR (Figure 6C). In vitro decidualization of HESCs was stimulated with 8-Br-cAMP and MPA. Two decidual biomarkers (PRL and IGFBP1) were gradually increased during artificial induction of decidualization (0, 2, 4, 6 and 8 days) at mRNA level (Figure 6D). Furthermore, the RPA2 expression was examined during in vitro decidualization process. The results suggested that RPA2 expression reduced significantly in HESCs during artificial induction of decidualization over time (Figure 6E, F).

Discussion

Although the development of ART has got great progress over the past decades, numbers of infertile women is still experiencing embryo implantation failure frequently during ART, so the embryo implantation rate remains not satisfactory [19, 20]. Inadequate endometrial receptivity is believed as a key factor contributes to implantation failure [21, 22]. However, there is no clear explanatory mechanism for insufficient endometrial receptivity. Raising evidences suggest that impaired decidualization of endometrial stromal cells in RIF patients plays a blocking part in embryo implantation [7, 23, 24]. In addition, proper proliferation capacity of endometrial stromal cells during implantation is necessary. In this study, we found that the proliferation-related gene RPA2 reduced in endometrial tissues of RIF patients compared with the healthy women and affected endometrial stromal cell proliferation and decidualization. Therefore, it is essential to explore the role of RPA2 in endometrial decidualization.

It is commonplace to find significantly differential genes of RIF through high-throughput sequencing analysis and bioinformatics analysis. Zhou et al reported that EHD1 was significantly higher in mid-secretory endometrium of RIF patients than the control group using an RNA-seq analysis, further discovered that EHD1 impaired decidualization by regulating the Wnt4/β-catenin signaling pathway in RIF [25]. Another study demonstrated that ATF3 expression was obviously downregulated in the endometrium of RIF patients through RNA-seq analysis and ATF3 deficiency weakened the proliferative–secretory phase transition and decidualization in RIF women [6]. In our previous study, we filtered differentially expressed circRNAs, miRNAs and mRNAs by seeking public database and bioinformatics analysis, and constructed hsa_circ_0038383/miR-196b-5p/HOXA9 axis which provided a novel insight into exploring molecular mechanism [16]. In this study, we retrieved the expression profiles from GEO databases and filtered the DEGs between RIF and the fertile
control group. The DEmRNAs related biological progresses and pathways were performed through GO and KEGG analysis. The results suggested that the DEGs were primarily involved in DNA replication or repair and cell apoptosis or cycle. Subsequently, four downregulated genes (RFC4, PCNA, PLK4 and RPA2) were considered as the initial target genes. There is no RFC4-related research in embryo implantation. McCoy et al. found that the aberrant expression of PLK4 caused tripolar mitosis and aneuploidy in embryo pre-implantation and further led to RIF [18]. For PCNA and RPA2, there is almost no research in RIF. Therefore, it is necessary to explore their roles in RIF. Besides, we detected their expression in endometrial tissues of RIF women compared with the control group. The results revealed that the expression of RPA2, PCNA and PLK4 were evidently higher in endometrial tissues of RIF than the control group, but there was no difference in RFC4 between two groups. Proliferating Cell Nuclear Antigen (PCNA), as a maker of proliferation, was uncovered more interest pre-test results, and further in-depth molecular biology experiments on PCNA needed to be executed in the future study. There have been a study reported PLK4 with abnormal expression in RIF patients than the healthy women [17]. Thence, RPA2 was selected our target object.

RPA2 is a member of the Replication Protein A complex composed by RPA1, RPA2 and RPA3 [26]. RPA complexes play a unique role in replication, meiotic recombination and apoptosis [11]. The silencing of RPA1 enhanced radiosensitivity via blocking RAD51 to the DNA damage site, and further contributed to G2/M cell cycle arrest and impaired cell proliferation in nasopharyngeal cancer [27]. RPA3 upregulation promoted cells proliferation is associated with poorer patient survival in hepatocellular carcinoma [28]. Elevated RPA2 expression facilitated the DNA structure formation and cell proliferation [29, 30]. RPA2 as a key factor related to proliferation, its role in endometrium during embryo implantation remains unclear.

Proper transition from the proliferative to secretory phase and decidualization is essential for the successful establishment of pregnancy [5, 31]. Appropriate proliferation of endometrial stromal cells is required for decidualization and further embryo implantation failure. For instance, HOXA10 as a marker of endometrial receptivity, its decreased expression could inhibit the proliferation of stromal cells during decidualization, and reduced fertility happened in HOXA10-mutant mice [32, 33]. Protopanaxadiol promoted the proliferation and the expression of decidualization-related genes in decidual NK cells, and further contributed to fertility and prevented pregnant mice from miscarriage [34]. In this study, RPA2 was signficantly higher in the endometrium of RIF patients than the control group. And the expression of RPA2 was gradually decreased under treating with 8-Br-cAMP and MPA, and the low expression in decidual tissues was proved in clinical specimens. These results could be explained as follow: the implantation phase of the endometrium requires not only the decidualization of the endometrium, but also low proliferation ability. In RIF patients, RPA2 upregulation caused proliferative intima unable to transition to decidualization, and further led to embryo implantation failure.

**Conclusion**

In summary, our study identified RPA2 as hub gene involved in RIF based on GEO database and a series of comprehensive analyses of bioinformatics. The upregulation of RPA2 in the endometrium of RIF patients inhibited decidualization, and ultimately to implantation failure. However, further in-depth molecular biology experiments on RPA2 related signaling pathway in RIF and decidualization need to develop. These findings
revealed that aberrant RPA2 in the endometrium could result in RIF, and offers novel insight into the molecular mechanism of RPA2.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| GEO          | Gene Expression Omnibus |
| RIF          | Recurrent implantation failure |
| HESCs        | Human endometrial stromal cells |
| IVF-ET       | in vitro fertilization-embryo transfer |
| ART          | Assisted reproductive technology |
| WOI          | Window of implantation |
| DEGs         | differentially expressed genes |
| RT-qPCR      | Real-time quantitative polymerase chain reaction |
| GO           | Gene Ontology |
| KEGG         | Kyoto Encyclopedia of Gene and Genome |
| PPI          | Protein-protein interaction |
| STRING       | the Search Tool for the Retrieval of Interacting Genes database |

**Declarations**

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**Author contributions**

Huishan Zhao: conception, idea, design of the study, experiments and writing the original draft; Mingwei Yu and Jianxiang Cong: data collection and curation, experiments, review and editing the draft; Gang Chen: methodology, data analysis and visualization; Xuemei Liu and Hongchu Bao: supervision, project administration, funding acquisition and paper finalization. All authors read and approved the final manuscript.

**Conflicts of interest**

The authors declare that there is no conflict of interest.

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Availability of data and materials

All data used during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All participants signed informed consent forms approved by the Institutional Review Board of Yantai Yuhuangding Hospital (reference number 2019-121) before performing any study-related procedure.

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### Tables

#### Table 1 Basic information of the three microarray datasets from GEO

| Data source   | Platform   | Tissue       | Sample size (RIF/control) | Author      | Year | Region     | RNA type |
|---------------|------------|--------------|---------------------------|-------------|------|------------|----------|
| GSE58144      | GPL15789   | Endometrium  | 43/72                     | van Hooff   | 2015 | Netherlands| mRNA     |
| GSE111974     | GPL17077   | Endometrium  | 24/24                     | Sezerman    | 2018 | Turkey     | mRNA     |

#### Table 2 Clinical characteristics of women with RIF and the control group recruited in the present study
| Variables                          | Control groups (n = 24) | RIF groups (n = 24) | p value |
|-----------------------------------|-------------------------|---------------------|---------|
| Age (years)                       | 30.83 ± 3.69            | 31.00 ± 3.65        | 0.88\(^a\) |
| BMI (kg/m\(^2\))                  | 21.29 ± 1.83            | 21.97 ± 2.48        | 0.29\(^a\) |
| FSH (mIU/ml)                      | 7.37 ± 2.24             | 7.13 ± 1.84         | 0.68\(^a\) |
| LH (mIU/ml)                       | 4.85 ± 2.15             | 4.47 ± 2.36         | 0.56\(^a\) |
| Estradiol (pg/ml)                 | 36.33 ± 14.17           | 34.96 ± 15.76       | 0.75\(^a\) |
| AMH (ng/ml)                       | 3.17 ± 0.73             | 3.10 ± 0.96         | 0.78\(^a\) |
| Infertility duration (years)      | 3.90 ± 1.58             | 3.53 ± 2.03         | 0.48\(^a\) |
| Number of embryo transfer cycles  | 1 (19/24, 79.2%)        | 3 (12/24, 50.0%)    | <0.001\(^b\) |
|                                   | 2 (5/24, 20.8%)         | 4 (6/24, 25%)       |         |
|                                   |                         | 5 (4/24, 16.7%)     |         |
|                                   |                         | 6 (2/24, 8.3%)      |         |
| Number of embryos per transfer    | 1.58 ± 0.72             | 1.54 ± 0.59         | 0.83\(^a\) |
| Average score of embryos transferred | 7.79 ± 0.41             | 7.83 ± 0.38         | 0.72\(^a\) |

\(^a\)Student t-test; \(^b\)fisher’s exact test.

Data presented as mean ± SD. \(P < 0.05\) was considered statistically significant.

### Table 3 Clinical characteristics of 30 women in different phases

| Variables                          | Proliferative phase (n = 10) | Secretory phase (n = 10) | Decidual tissues (n = 10) | p value |
|-----------------------------------|-----------------------------|--------------------------|---------------------------|---------|
| Age (years)                       | 31.50 ± 2.92                | 31.60 ± 3.20             | 34.30 ± 6.77              | NS \(^c\) |
| BMI (kg/m\(^2\))                  | 23.43 ± 3.79                | 24.07 ± 4.88             | 23.82 ± 3.87              | NS \(^c\) |
| FSH (mIU/ml)                      | 6.44 ± 3.52                 | 6.36 ± 3.38              | 6.79 ± 3.03               | NS \(^c\) |
| LH (mIU/ml)                       | 3.64 ± 2.16                 | 3.77 ± 1.82              | 3.52 ± 1.64               | NS \(^c\) |
| Estradiol (pg/ml)                 | 31.84 ± 12.50               | 30.99 ± 9.31             | 30.44 ± 11.02             | NS \(^c\) |
| AMH (ng/ml)                       | 3.04 ± 1.27                 | 3.28 ± 0.92              | 3.31 ± 1.50               | NS \(^c\) |
| Gestational week (weeks)          |                             |                          | 6.60 ± 0.84               | NA      |
One-way ANOVA

NS: No Significant; NA: No Available.

Table 4 Primer sequences for RT-qPCR

| Genes   | Forward primer (5'→3') | Reverse primer (5'→3') |
|---------|------------------------|------------------------|
| RPA2    | GCAGGCCACCTGAGATCTTT   | ATAGGTGCTCTCCCTGCTGA   |
| PLK4    | CTTTATCACCTCTCTCTCTTC  | CCAAGTCTTCATTTTGTAACC  |
| RFC4    | GCGGAAACCTGAGGAAGGAGCC | TGGCAGCTACTCTCGATCTTTG |
| PRL     | TTAGGGGCATGGAGCTGATA   | GCACTTCAGGACTCTGGGATATA |
| IGFBP1  | TTAGCCAAGGCACAGGAGAC   | CCCATTCAAAGGTAGACGC    |
| GAPDH   | CATGTTCTGCTATGGGTGGA   | GGCATGGACTGGTGTCATGAG  |

Figures

**Figure 1**

Identification of DEGs in RIF. (A) Volcano map of the differentially expressed mRNAs from the GEO microarray GSE58144. (B) Volcano map for all mRNAs in GSE111974. (C) Venn plots of downregulated overlapping
DEGs. (D) Venn plots of upregulated overlapping DEGs. (E) The heat map of top 20 downregulated and upregulated DEGs in the integrated microarray analysis.

Figure 2

GO and KEGG analyses of DEGs. (A) GO analysis of upregulated DEGs. (B) KEGG pathway analysis of upregulated DEGs. (C) GO analysis of downregulated DEGs. (D) KEGG pathway analysis of downregulated DEGs.
Figure 3

Construction of PPI network and module analysis. (A) The PPI network of 236 DEGs. Blue nodes indicate the downregulated DEGs and red nodes represent the upregulated DEGs. (B) Module 1 of PPI network. (C) Module 2 of PPI network. (D) GO circle plot of four target genes (RFC4, PCNA, PLK4 and RPA2). (E) KEGG circle plot of four target genes (RFC4, PCNA, PLK4 and RPA2). ***p < 0.001.
Figure 4

The expression RFC4 and PLK4 in endometrium of RIF patients compared with the healthy women and the endometrium of different phase. The PLK4 mRNA (A) and RFC4 mRNA (B) in endometrial tissues of RIF patients and the control group. The mRNA level of PLK4 (C) and RFC4 (D) in the proliferative, secretory and decidual phases of endometrial tissues using qPCR. P: proliferative phase; S: mid-secretory phase; D: decidual phase.

Figure 5

Aberrant expression of RPA2 in the endometrium. (A) Immunohistochemical staining of RPA2 in the endometrium of mid-secretory phase from RIF patients (n = 5) and control group (n = 5). (B) Representative western blotting images of RPA2 in endometrium from RIF (n = 5) and control (n = 5). Quantitative densitometry analysis suggested that RPA2 level were upregulated in RIF patients (n = 24) compared with the
control (n = 24). (C) RPA2 mRNA in RIF (n = 24) endometrium was higher than the control (n = 24) detecting by qPCR. MS: mid-secretory. *p < 0.05, **p < 0.01.

Figure 6

**Decreased RPA2 expression level upon decidualization.** (A) Immunohis to chemistry analysis of RPA2 in the proliferative (n = 5), secretory (n = 5) and decidual phases (n = 5). Its expression level was gradually reduced. (B) The expression level of RPA2 in the proliferative (n = 10), secretory (n = 10) and decidual phases (n = 10). (C) RPA2 expression in original endometrial epithelial cells (n = 3) and stromal cells (n = 3). (D) The mRNA level of PRL and IGFBP1 in HESCs treated with 0.5 mM 8-Br-cAMP and 1 μM MPA(0, 2, 4, 6 and 8 days) in vitro. (E) The mRNA level of RPA2 was decreased during decidualization (0, 2, 4, 6 and 8 days) in vitro using qPCR. (F) The protein level of RPA2 was decreased during decidualization (0, 2, 4, 6 and 8 days) in vitro using western blotting. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.001.