REPRODUCTIVE PHYSIOLOGY AND DISEASE

Upregulated RPA2 in endometrial tissues of repeated implantation failure patients impairs the endometrial decidualization

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Abstract

Purpose To investigate the expression and underlying mechanism of RPA2 in endometrium of patients with repeated implantation failure (RIF).

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 a 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. Furthermore, RT-qPCR was used to detect changes in the decidual biomarkers after transfection of RPA2 overexpression vector in human endometrium stromal cell (HESC).

Results RPA2 was significantly upregulated in the mid-secretory endometrium of patients with RIF. As a proliferation-related gene, RPA2 was obviously higher expressed at proliferative phase during the normal menstrual cycles. Moreover, the downregulation of RPA2 was discovered during decidualization of HESC. Furthermore, RPA2 overexpression impaired decidualization by inhibiting the expression of prolactin (PRL) and insulin-like growth factor-binding protein 1 (IGFBP1).

Conclusions Our finding indicated that aberrant upregulation of RPA2 attenuated decidualization of HESC in RIF women and provided new potential therapeutic targets.

Keywords RPA2 · Repeated implantation failure (RIF) · Decidualization · Human endometrial stromal cell (HESC)

Abbreviations

GEO	Gene Expression Omnibus
RIF	recurrent implantation failure
HESC	human endometrial stromal cell
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

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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 undergoes decidualization in preparation for implantation [5]. The establishment of the window of implantation (WOI) phase and successful clinical pregnancy requires proper transition from the proliferative to secretory phase. The inappropriate transition between these two phases and decidualization deficiency are apparent 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 mechanism of affecting decidualization contributes to understanding the initiation and progression of RIF.

To elucidate how the endometrial gene expression profile differs between women with RIF and controls, we download the mRNA expression profiles (GSE58144 and GSE111974) of endometrium tissues from the Gene Expression Omnibus (GEO) database. After screening and verification, RPA2 was selected as a 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]. RPA2 hyperphosphorylation caused by DNA damage-induced involves in cell cycle arrest and further influences cell proliferation [12]. Nevertheless, it is unclear whether RPA2-mediated growth is responsible for the proliferative-secretory phase transition, embryo implantation during decidualization, or the pathogenesis of RIF. In this study, we compared the expression of RPA2 in mid-secretory endometrium between RIF patients and healthy women. In addition, we examined the expression of RPA2 in proliferative phases and secretory phases of the endometrium during the menstrual cycle and in the decidual tissue of early pregnant women. Also, the role of RPA2 in decidualization of stromal cells was investigated. 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 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 is the GPL17077 (Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray 039381), includes 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 is 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 is conducted to find key pathways the DEGs participated in RIF [14]. The associated data is 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 is conducted to visualize the interaction among the differentially expressed proteins based on the Search Tool for the Retrieval

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

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 are appraised by cytoHubba plugin in cytoscape. Also, MCODE module is 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 2022-018) before performing any study-related procedure.

Twenty-four 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 and uterus-related diseases achieved a clinical pregnancy after their first or second embryo transfer was enrolled. The exclusion and inclusion criteria followed in our previous study [16]. The characteristics of these

patients are listed in Table 2. Simultaneously, 20 infertility women without uterus-related diseases and 10 healthy early pregnant women with induced abortion due to abnormal embryonic development were selected according to 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 are shown in Table 3.

Cell culture and primary cells isolation

HESC (human endometrial stromal cell) is an immortalized cell line, which is a kind gift from Professor Haibin Wang (School of Medicine, Xiamen University, Xiamen, China). HESC were cultured in phenol red-free DMEM/F12 (Gibco BRL/Invitrogen, Carlsbad, CA, USA) supplied with 10% charcoal stripped fetal bovine serum (Biological Industries, Israel) [17].

Primary endometrial epithelial cells and stromal cells were isolated from the mid-secretory phase endometrial

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%) 2 (5/24, 20.8%)	3 (12/24, 50.0%) 4 (6/24, 25%) 5 (4/24, 16.7%) 6 (2/24, 8.3%)	<0.001[b]
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

[c]One-way ANOVA

NS, no significant; *NA*, no available

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, the stromal cells were separated from the epithelial cells through a 40-μm filter.

In vitro decidualization

HESC were treated with 0.5 mM 8-Br-cAMP (Sigma-Aldrich, St. Louis, Mo, USA) and 1 μM medroxyprogesterone acetate (MPA, MedChemExpress LLC, NJ, 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 examining cell morphology.

RPA2-overexpression transfection

RPA2-overexpression and control vector plasmids were purchased from OriGene Technologies (Rockville, MD, USA). One microgram of RPA2-overexpression or control vector was transfected into HESC, respectively, using the X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

RNA isolation and RT-qPCR

Total RNA was extracted from cells and tissue samples using Trizol reagent following the 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 2 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The primer sequences are showed 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 μg) were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. After blocking with 5% skimmed milk for 1 h at room temperature, the membranes were incubated with antibodies against RPA32/RPA2 (1:1000; 35869T; Cell Signaling Technology) and GAPDH antibody (1:2000; D110016-0100; Sangon Biotech). The next day, membranes were incubated with a HRP-conjugated goat anti-rabbit IgG h+1 secondary antibody (1:5000; abs20040ss; absin). The bands are 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. Four-mm-thick tissue sections were got from the paraffin tissue block. First, the tissue sections were grilled in oven with 65 °C for 1 h. 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 (breaking aldehyde bonds, methylol and hydroxyl cross-linked protein) 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% H_2O_2 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). The next day, the sections were washed with PBS and incubated with secondary antibody, and 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.

Table 4 Primer sequences for RT-qPCR

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
RPA2	GCAGGCCACCTGAGATCTTT	ATAGGTGCTCTCCCTGCTGA
PLK4	CCTTATCACCTCCTCCTTC	CCAAGTCCTTCATTTGTAACC
RFC4	GCGGAAACCTGAGGAACGAGCC	TGGCAGCTACTCCTCGATCCTTG
PRL	TAGAGGGCATGGAGCTGATA	GCACTTCAGGAGCTTGAGATAA
IGFBP1	TTAGCCAAGGCACAGGAGAC	CCCATTCCAAGGGTAGACGC
GAPDH	CATGTTCGTCATGGGTGTGAA	GGCATGGACTGTGGTCATGAG

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). $p<0.05$ 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) is shown in Table 1. A total of 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 (RIF vs Control) (Fig. 1A). Also, we screened out 4508 DEGs (1972 upregulation and 2536 downregulation) with $p<0.01$ from GSE111974 (Fig. 1B) using the same data processing method. Subsequently, 162 overlapping downregulated DEGs (Fig. 1C) and 74 overlapping upregulated DEGs (Fig. 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 (Fig. 1E).

GO and KEGG enrichment analysis

To explore the biological functions and pathways of DEGs in RIF, GO annotation analysis was conducted using the DAVID online analysis tool. The biological process, cellular component, and molecular function terms of upregulated DEGs are shown in Fig. 2A. As shown in Fig. 2B, KEGG pathway analysis revealed that the upregulated DEGs in RIF significantly were enrich in "apoptosis," "NF-kappa B signaling pathway," "AMPK signaling pathway," "cell adhesion molecules," and other related signaling pathways. The downregulated DEGs were significantly enrich in DNA replication- or repair-related biological functions (Fig. 2C). As shown in Fig. 2D, KEGG pathway analysis of the downregulated DEGs was conducted in RIF, of which the critical pathways were "DNA replication," "DNA repair," "metabolic pathways," and "cell cycle." Therefore, the DEGs were mainly associated with DNA replication or repair.

PPI network construction

Based on the STRING database, a PPI network was established for further investigation of the interaction
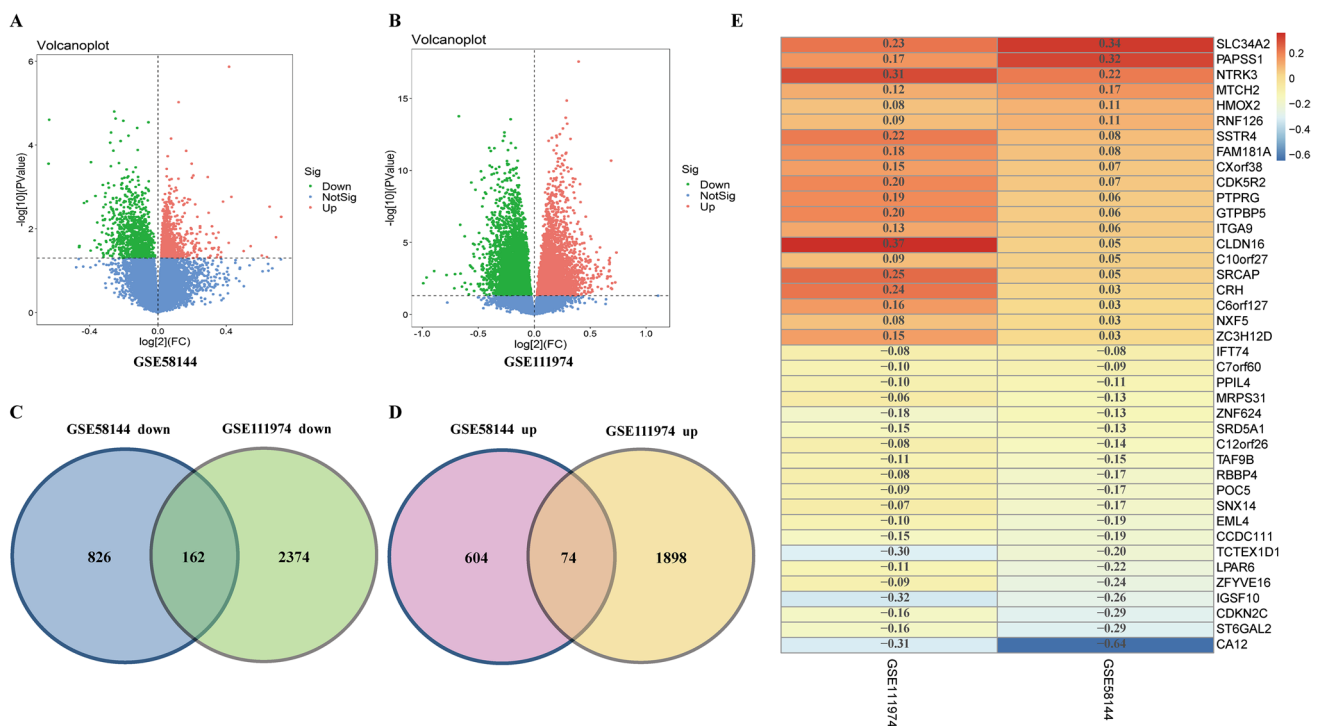

Fig. 1 Identification of DEGs in RIF. **A** Volcano map of the differentially expressed mRNAs from the GEO microarray GSE58144. **B** Volcano map for all mRNAs in GSE111974. **C** Venn plots of downregulated overlapping DEGs. **D** Venn plots of upregulated overlapping DEGs. **E** The heat map of top 20 downregulated and upregulated DEGs in the integrated microarray analysis
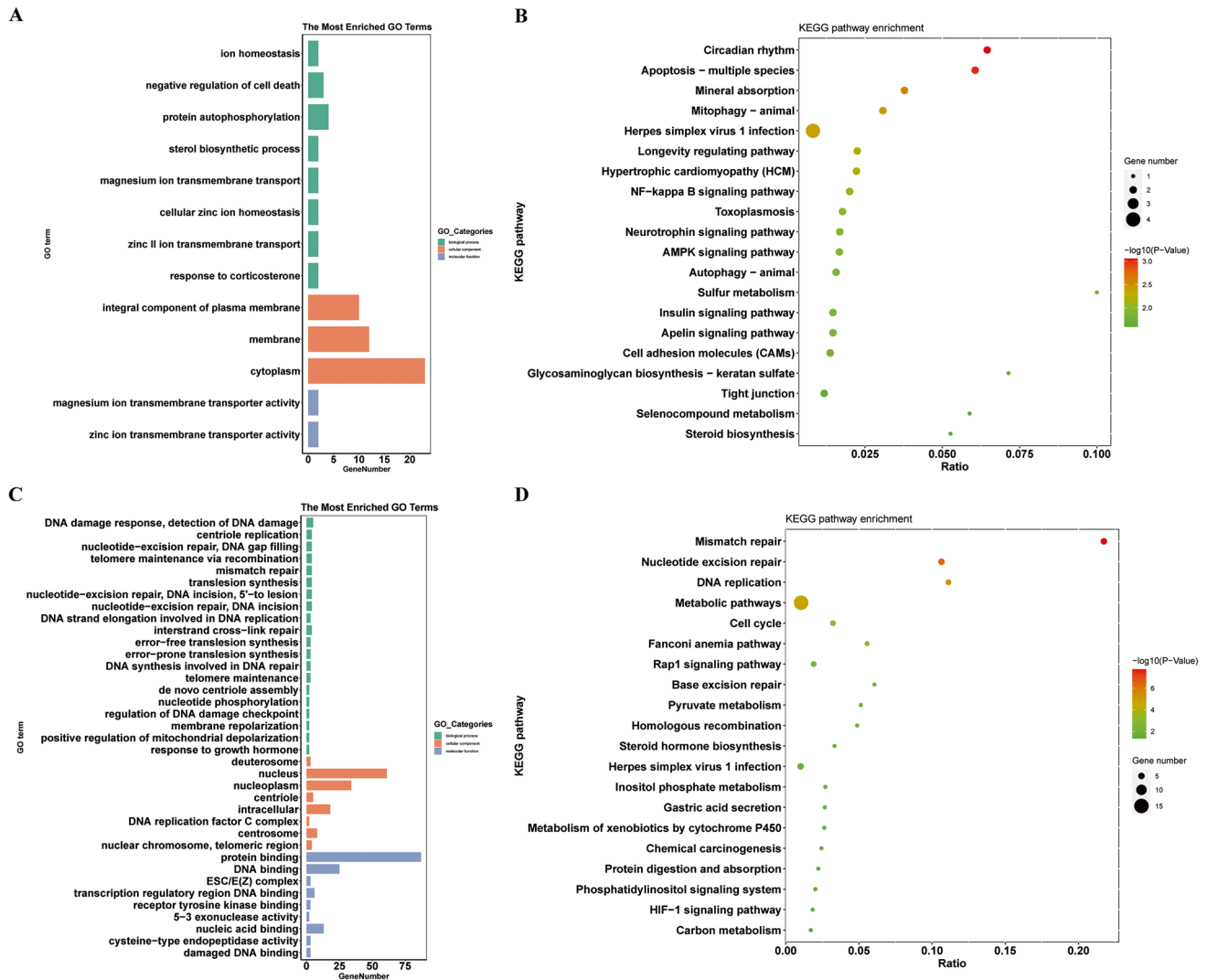
Fig. 2 GO and KEGG analyses of DEGs. **A** GO analysis of upregulated DEGs. **B** KEGG pathway analysis of upregulated DEGs. **C** GO analysis of downregulated DEGs. **D** KEGG pathway analysis of downregulated DEGs

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 (Fig. 3A). Four DEGs with degree > 10 were identified as hub genes, which were RFC4, PCNA, PLK4, and RPA2, respectively. Subsequently, we explored the significant clusters in this PPI network; two clusters closely connected were discovered (Fig. 3B, C). Interestingly, four target genes (RFC4, PCNA, PLK4, and RPA2) were all uncovered in the second cluster (Fig. 3C), which demonstrated that these four genes not only had high degree, but also held close relationship among them. Furthermore, we investigated the biological functions and signaling pathways involved in these four hub genes. As shown in the GO circle plot

and KEGG circle plot of Fig. 3D and E, they were mainly associated with DNA replication or repair and cell cycle.

Hub gene validation

Aiming to confirm the four candidate hub genes in RIF, we detected their expression in endometrial tissues of RIF women compared with the control group. The results illustrated that PCNA and RPA2 were upregulated in RIF, which was consistent with the predicted results. However, RFC4 and PLK4 with high expression in RIF were inconsistent with the GEO data (Fig. 4A, B). In addition, PLK4 was downregulated in the endometrium of different phases (proliferative phase, mid-secretory phase, and decidual tissues), whereas there was no difference in RFC4 among the
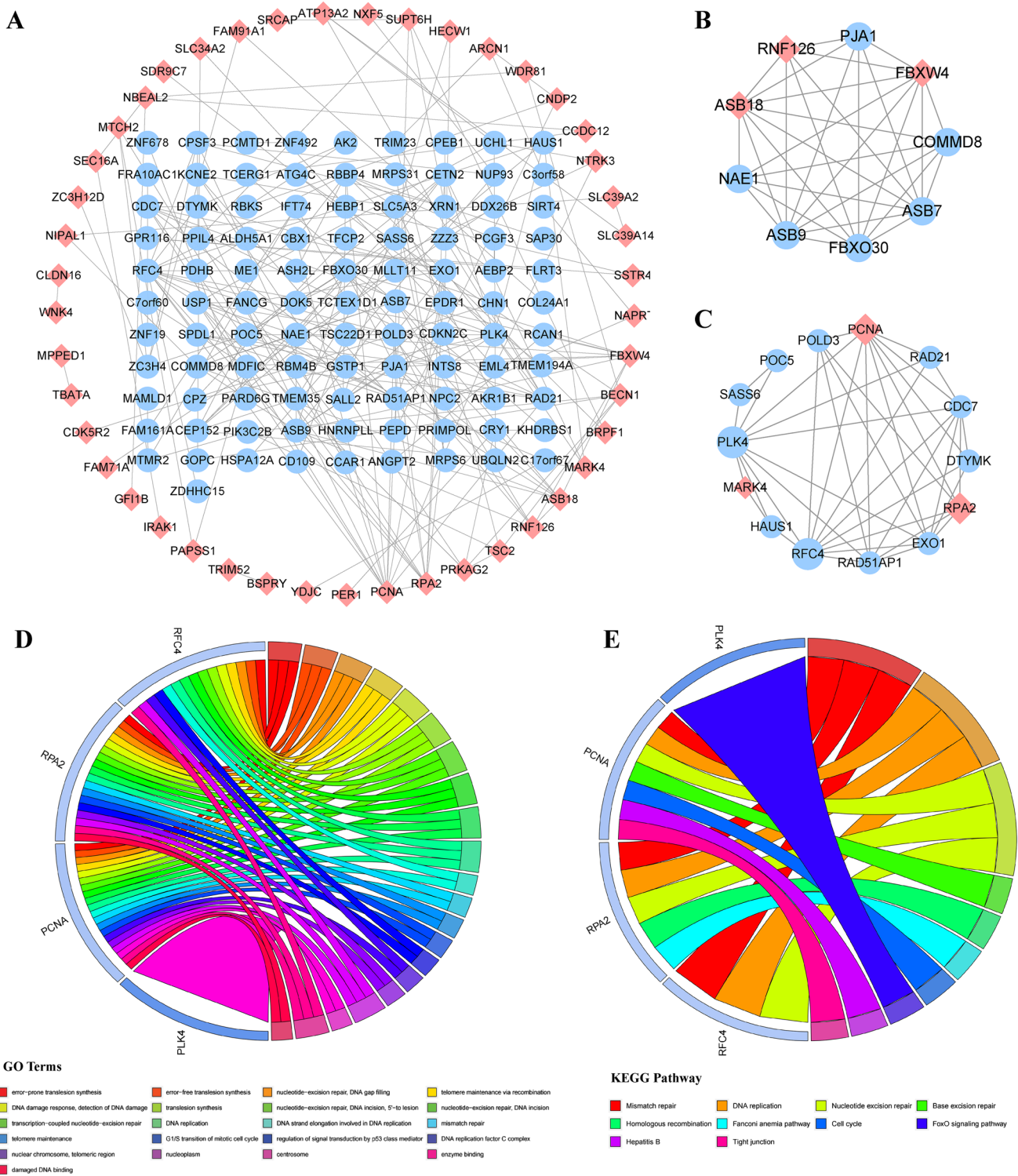
Fig. 3 Construction of PPI network and module analysis. **A** The PPI network of 236 DEGs. Blue nodes indicate the downregulated DEGs and red nodes represent the upregulated DEGs. **B** Module 1 of PPI network. **C** Module 2 of PPI network. **D** GO circle plot of four target genes (RFC4, PCNA, PLK4, and RPA2). **E** KEGG circle plot of four target genes (RFC4, PCNA, PLK4, and RPA2). $^{***}p < 0.001$

three phases (Fig. 4C, D). Interestingly, we found more meaningful results of PCNA in RIF, which could be displayed as an independent study, but not in this research. Therefore, RPA2 was selected as the key research object of this study.

Aberrant expression of RPA2 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 mid-secretory phase endometrium, and RPA2 was expressed in both stromal cells and epithelial cells (Fig. 5A). Moreover, Western blotting was applied to validate that RPA2 had adequate expression in the endometrium of RIF patients compared with the control group (Fig. 5B), which was consistent with the qPCR results (Fig. 5C). All the sections were in the mid-secretory phase.

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

Due to the fact that decidualization of stromal cells is a key step for the uterus to receive embryos, and decidualization

dysregulation may lead to implantation failure, we investigated the role of RPA2 in the decidualization of stromal cells. To explore the pathophysiological significance of RPA2 during early pregnancy, we detected the expression pattern of RPA2 using immunohistochemistry and qPCR. The results suggested that RPA2 protein abundance gradually decreased during the menstrual cycle (Fig. 6A), and its tendency at protein level was consistent with mRNA (Fig. 6B). Moreover, higher expression of RPA2 was observed in endometrial epithelial cells than stromal cells, and the differential expression of RPA2 in these two types of cells is further tested and verified in primary endometrial epithelial cells and stromal cells by qPCR (Fig. 6C). *In vitro* decidualization of HESC 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 (Fig. 6D). Furthermore, the RPA2 expression was examined during *in vitro* decidualization process. The results suggested that RPA2 expression reduced significantly in HESC during artificial induction of decidualization over time (Fig. 6E, F). We also investigate the RPA2 expression in primary HESC during *in vitro* decidualization. Interestingly, the expression trend of RPA2 in primary HESC and HESC immortalized cell line is consistent during *in vitro* decidualization (Fig. S1).

Fig. 4 The expression RFC4 and PLK4 in endometrium of RIF patients compared with the healthy women and in the endometrium of different phases. 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
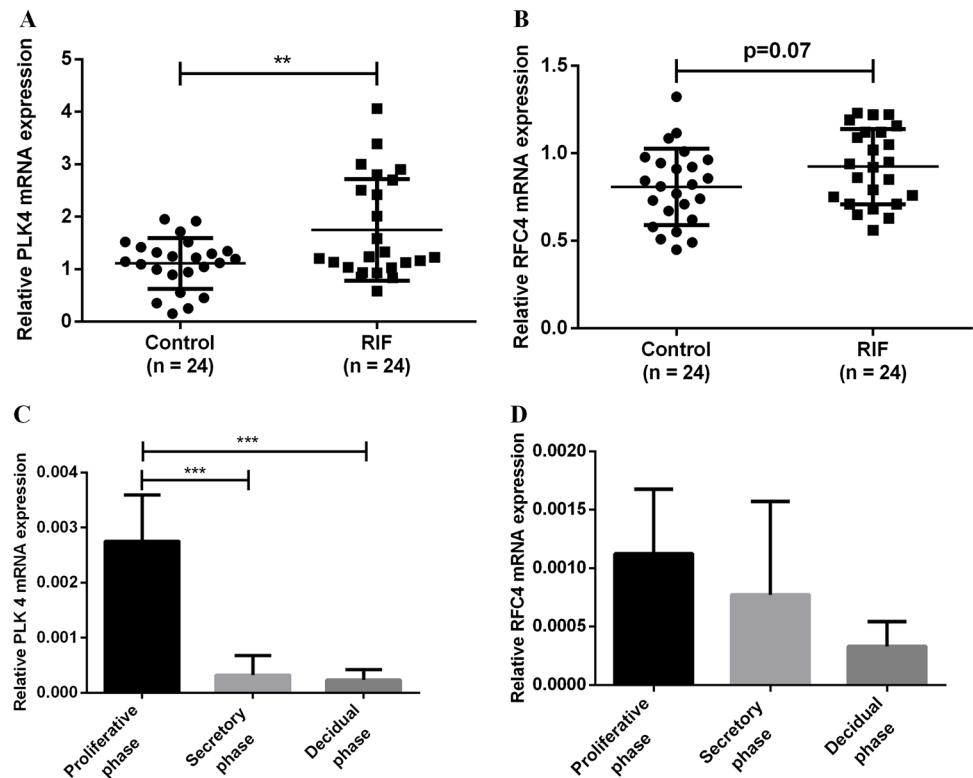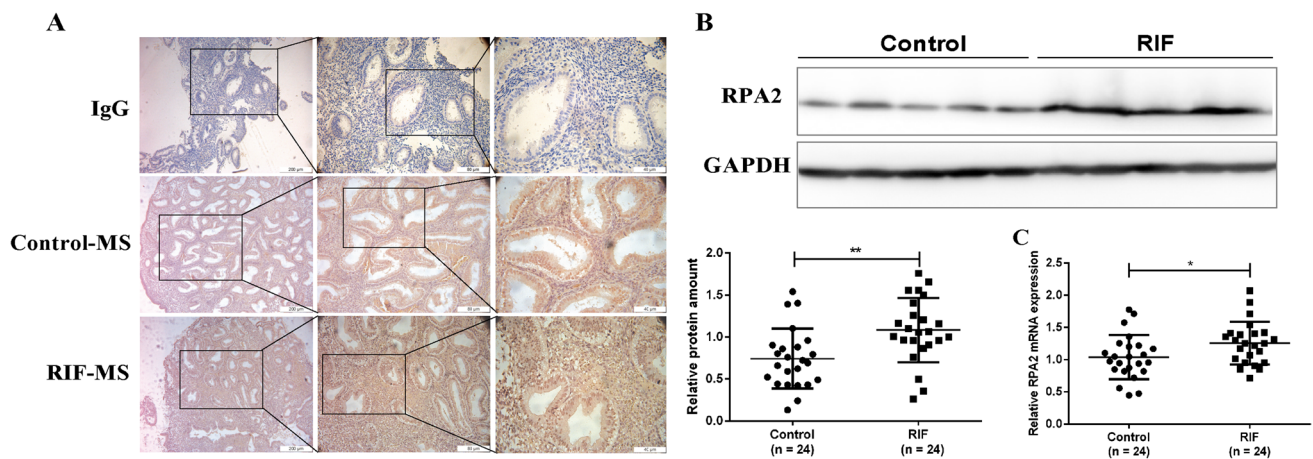
Fig. 5 Aberrant expression of RPA2 in the endometrium. **A** Immunohistochemical staining of RPA2 in the endometrium of mid-secretory phase from RIF patients ($n = 5$) and control group ($n = 5$). **B** Representative Western blotting images of RPA2 in endometrium from RIF ($n = 5$) and control ($n = 5$). Quantitative densitometry analysis suggested that RPA2 level was 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 ($n = 24$) detected by qPCR. MS, mid-secretory. $^{*}p < 0.05$, $^{**}p < 0.01$

RPA2 overexpression impairs the decidualization of HESC

The reduced expression of RPA2 during decidualization encouraged us to explore its potential biological mechanisms. As shown in Fig. 7, when RPA2 was overexpressed in HESC, the mRNA level of PRL and IGFBP1 was substantially decreased on the 2th and 4th day of decidualization *in vitro*.

Discussion

Although the development of ART has made great progress over the past decades, numbers of infertile women are still experiencing embryo implantation failure frequently during ART, so the embryo implantation rate remains unsatisfactory [18, 19]. Inadequate endometrial receptivity is believed as a key factor contributing to implantation failure [20, 21]. However, there is no clear explanatory mechanism for insufficient endometrial receptivity. Growing evidence suggests that impaired decidualization of endometrial stromal cells in RIF patients plays a blocking part in embryo implantation [7, 22, 23]. In this study, we found that the proliferation-related gene RPA2 was increased in endometrial tissues of RIF patients compared with healthy women and affected endometrial stromal cell proliferation and decidualization. Therefore, it was essential to explore the role of RPA2 in endometrial decidualization.

It is commonplace to find significantly different genes in RIF patients through high-throughput sequencing analysis and bioinformatics analysis. Zhou *et al.* reported that EHD1 was significantly higher in the mid-secretory endometrium of RIF patients than the control group using RNA-seq analysis, and further discovered that EHD1 impaired decidualization by regulating the Wnt4/β-catenin signaling pathway in RIF [24]. Another study demonstrated that ATF3 expression was obviously downregulated in the endometrium of RIF patients through RNA-seq analysis and that 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 databases 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 DEGs (RFC4, PCNA, PLK4, and RPA2) were considered the initial target genes. There was no RFC4-related research in embryo implantation. PLK4 was reported as a key gene, regulatory factor, and drug target gene of RIF, which was downregulated in RIF [25]. In addition, the abnormal expression of PLK4 in human pre-implantation embryo could lead to tripolar mitosis and aneuploidy, which would result in IVF failure [26]. In our study, we detected the expression of these four initial target genes in the RIF group and the control group. The difference of RFC4 between RIF and the control group was not significant. The expression of PLK4 was inconsistent with the GEO database, and the role
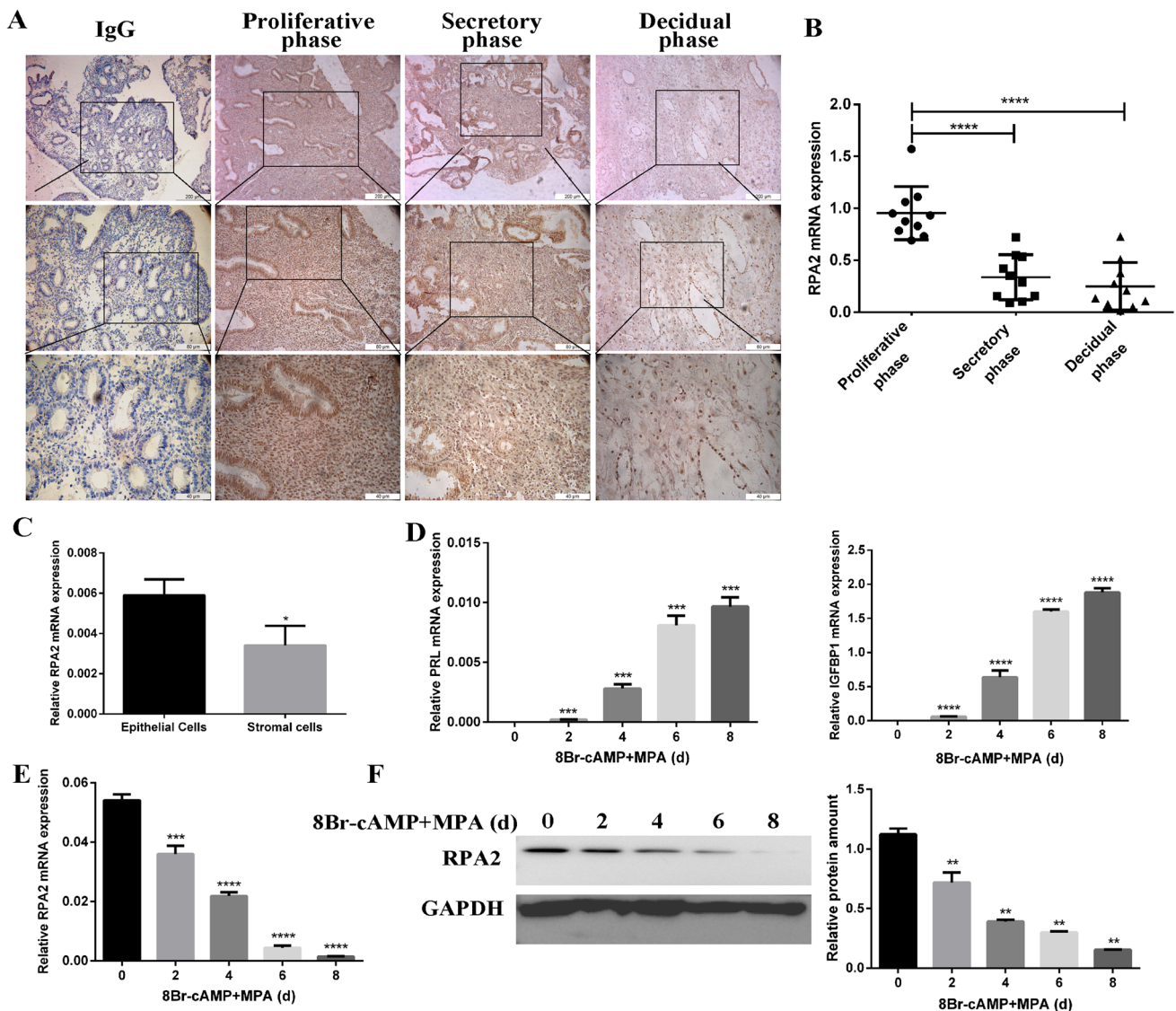
Fig. 6 Decreased RPA2 expression level upon decidualization. **A** Immunohistochemistry analysis of RPA2 in the proliferative ($n = 5$), secretory ($n = 5$), and decidual phases ($n = 5$). Its expression level was gradually reduced. **B** The expression level of RPA2 in the proliferative ($n = 10$), secretory phases ($n = 10$), and decidual tissues ($n = 10$). **C** RPA2 expression in original endometrial epithelial cells ($n = 3$) and stromal cells ($n = 3$). **D** The mRNA level of PRL and IGFBP1 in HESC 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. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.001$

of PLK4 RIF had been reported. 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 are needed to be executed in the future study. Finally, we reported only RPA2 in the remaining sections, discarding results from RFC4, PLK4, and PCNA.

RPA2 is a member of the replication protein A complex, composed by RPA1, RPA2, and RPA3 [27]. RPA complexes play an 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 [28]. RPA3 upregulation promoted cell proliferation and was associated with poorer patient survival in hepatocellular carcinoma [29]. Elevated RPA2 expression facilitated DNA structure formation and cell proliferation [30, 31]. The role of RPA2, a key factor related to proliferation, in endometrium during embryo implantation remained unclear.

Proper transition from proliferative phase to secretory phase and decidualization is essential for the successful
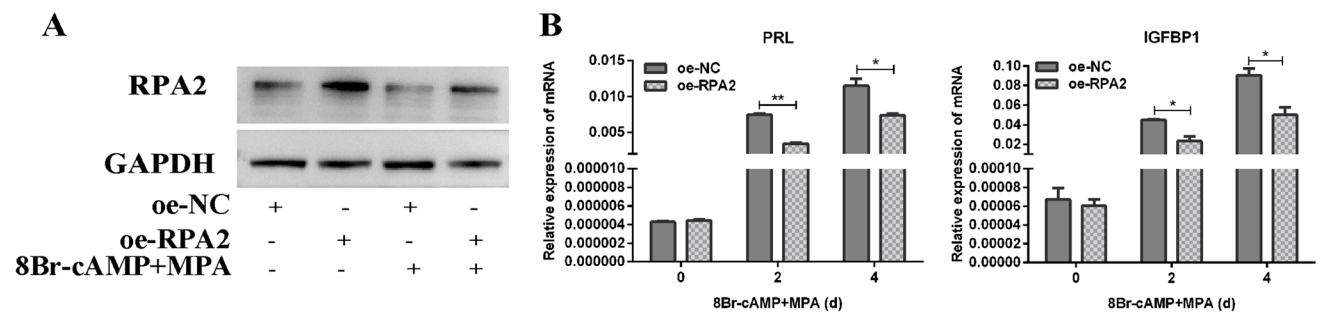
Fig. 7 RPA2 overexpression impaired HESC decidualization in vitro. **A** The protein expression of RPA2 in HESC transfected with oe-NC or oe-RPA2 and then treated with a decidualization stimulus for 2 days. **B** The expression of PRL and IGFBP1 in HESC transfected with oe-NC or oe-RPA2 and then treated with a decidualization stimulus for 2 and 4 days

establishment of pregnancy [5, 32]. Appropriate proliferation of endometrial stromal cells is required for decidualization and further embryo implantation. For instance, the decreased expression of HOXA10 as a marker of endometrial receptivity could inhibit the proliferation of stromal cells during decidualization, and induced fertility happened in HOXA10-mutant mice [33, 34]. Protopanaxadiol promoted cell proliferation and decidualization-related gene expression in decidual NK cells, which further contributed to fertility and prevented pregnant mice from miscarriage [35]. In this study, RPA2 was significantly higher in the endometrium of RIF patients than in the control group. And the expression of RPA2 was gradually decreased under treatment with 8-Br-cAMP and MPA, and the low expression in decidual tissues was proven in clinical specimens. Moreover, RPA2 overexpression inhibited *in vitro* decidualization of HESC. These results could be explained as follows: 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 that was unable to transition to decidualization, and further led to embryo implantation failure.

Conclusion

In summary, our study identified RPA2 as a hub gene involved in RIF based on the GEO database and a series of comprehensive bioinformatics. The upregulation of RPA2 in the endometrium of RIF patients inhibited decidualization and ultimately led to implantation failure. However, further in-depth molecular biology experiments on RPA2-related signaling pathway in RIF are needed to develop.

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Declarations

Conflict of interest The authors declare no competing interests.

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