Higher Follicular Fluid Progesterone Down-regulated HPGD and COX2 in Granulosa Cells via Suppressing NF- κ B Signaling in Endometriosis

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Research

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Abstract

Background: Luteinized unruptured follicular follicle syndrome (LUFS) is a special type of ovulatory dysfunction and a common cause of infertility. It is estimated that its prevalence is 13% ~ 73% in endometriosis patients. Increasing evidences prove that LUFS is one of the reasons for endometriosis-related infertility. Any alteration in FF components and GCs in endometriosis may influence the developing oocyte and ovulation. This study aimed to explore the effect of local elevated progesterone in follicular fluid (FF) on ovulation in endometriosis patients.

Methods: A Prospective study with matched pairs design was conducted at a reproductive medicine center between July 2017 and January 2018 in patients undergoing in vitro fertilization (IVF) or intracytoplasmic sperm injection treatment (ICSI), while granulosa tumor-like cell line KGN (Bena culture collection, China) was used as *in vitro* cell model. Alterations in follicular and peritoneal fluid (PF) components identified with metabolomics analyses; Differentially expressed genes in GCs identified with transcriptome analysis; Polymerase chain reaction (PCR), western blot, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence were used to determine the expression of progesterone, NF- κ B related genes, *HPGD* and *COX-2*; NF- κ B binding identified with chromatin immunoprecipitation (ChIP).

Results: Patients with endometriosis exhibited a significantly higher basal serum progesterone level, higher serum level of progesterone on trigger day and higher progesterone expression level in FF and PF. GCs from endometriosis patients revealed decreased expression of *HPGD*, *COX-2* and suppressed NF- κ B signaling, as manifested by decreased expressions of *IL1R1* and *IRAK3*. Similarly, progesterone treatment *in vitro* down-regulated *HPGD* and *COX2* expression and suppressed NF- κ B signaling in KGN cells in a dose dependent manner, as manifested by decreased expressions of *IL1R1*, *IRAK3*, reduced *plkB α /I κ B α* ratio and nucleus translocation of p65. TNF- α , by contrast, increased expression of *IL1R1*, *IRAK3*, *plkB α* , p65 and *HPGD* in KGN cells. Furthermore, one potential p65 binding site was identified in the promoter region of *HPGD* by chromatin immunoprecipitation.

Conclusion: Endometriosis showed repression of NF- κ B pathways and down-regulation of *HPGD* and *COX2*, which play important roles in the process of ovulation by participating in the metabolism of prostaglandin E2 (PGE2), in granulosa cells (GCs) due to elevated progesterone in FF.

Introduction

Endometriosis is a chronic gynecologic disorder affecting approximately 10% of women at reproductive age and 40–50% of infertile women [1, 2]. It is a complex syndrome manifested by chronic pelvic pain and infertility. The etiology of endometriosis is unknown. However, it is now generally accepted that the main causes are the repeated retrograde travel and survival of shed endometrial tissues in the lower abdominal cavity. Endometriosis-associated pelvic pain is currently managed by suppression of ovulatory menses and estrogen production, cyclooxygenase inhibitors, and surgical removal of pelvic lesions; whereas *in vitro* fertilization (IVF) is frequently used to overcome infertility.

LUFS is a syndrome of failed ovulation in which the follicle does not rupture, despite of the secondary ovulatory changes, such as peak luteinizing hormone (LH), rising progesterone (P), or endometrial secretion transformation [3]. LUFS is a special type of ovulatory menstruation and a common cause of infertility. The incidence of LUFS is 11–23% in natural menstrual cycle [4], and is higher in controlled ovarian stimulation cycle; when combined with endometriosis, the incidence of LUFS is 13–73% [5]. It seems that LUFS is one of the reasons for endometriosis-related infertility. However, the mechanism by which endometriosis causes LUFS is still to be investigated.

FF, which consists of a variety of substances, including hormones, immune cells, cytokines, enzymes, anticoagulants, electrolytes, reactive oxygen species, lipids, cholesterol and antioxidants, plays an important role in the growth and development of the follicle [6–8]. Any alteration in FF components may influence the developing oocyte [6]. The metabolic profile of FF in patients with endometriosis could reflect the metabolic changes in the systemic circulation or alternatively reflect the local inflammation due to endometriosis lesions in the ovary or peritoneal cavity [9]. GCs have also been proved to play an important role in follicular development [10]. In patients with endometriosis, abnormalities in GCs might impair oocyte maturation and lead to poor oocyte quality [11]. Interestingly, as the most neighboring cells to FF, granulosa cells and theca cells of the follicle cooperate with resident and infiltrating immune cells to produce paracrine mediators of ovulation [12, 13], many of which are also common in inflammatory responses. While whether and how alteration in FF components affects ovulation in endometriosis by interacting with GCs remain unclear.

In the present study, we analyzed the alteration in components of FF from patients with endometriosis compared with control by metabolic spectrum, aiming to find intrafollicular cytokines, steroids, or other small molecule chemicals that might account for the ovulatory dysfunction in endometriosis patients. The differentially expressed genes in corresponding GCs of endometriosis patients were also explored via RNA sequencing. Furthermore, we aimed to identify the mechanism involved in the impairment of ovulation in patients with endometriosis and provide potential therapeutic targets for their ovulation dysfunction.

Materials And Methods

Ethics approval

This study was approved by the medical ethics committee of the Women's Hospital of Zhejiang University School of Medicine (Ethics Lot number 20170021). We confirm that human study in our experiments was performed in accordance with relevant guidelines and regulations.

Study population

A total of 100 patients, who underwent IVF or intracytoplasmic sperm injection treatment in the reproductive medical center of the Women's Hospital of Zhejiang University School of Medicine during July 2017 and January 2018 were enrolled in this study. Of the 100 subjects, there were 50 with ovarian

endometriosis and 50 in the control group. Patients with ovarian endometriosis were confirmed by laparoscopy in our hospital. Inclusion criteria included regular menstrual cycle (23-35 days), unilateral endometrioma (≥ 5 cm) and normal ovarian reserve (serum anti-Mullerian hormone level of > 1 ng/mL, antral follicular count > 4 and basal serum follicle-stimulating hormone level of < 10 IU/L). Patients aged over 35 years, treated by steroid hormones, with pelvic inflammatory disease, endocrine diseases, unexplained infertility, with immune disease, or chromosomal abnormalities were excluded. Tubal blockage or male-factor infertility as two isolated causes comprised the control group.

Follicular Fluid and GC Isolation

During surgery, FF was collected using a new aspiration needle when the first and largest follicle of the ovary was punctured and with a flushed needle for the first follicle of the other ovary. Only FF samples free of blood contamination upon both visual and microscopic examination and that contained mature (MII) oocytes were analyzed. Samples were centrifuged at 2900 rpm for 10 minutes to remove cell debris before being stored. GCs samples was collected on the oocyte retrieval day according to the protocol [14]. Firstly, GCs were centrifuged at $340 \times g$ for 5 minutes. Then they were gathered from the pellet with Ficoll solution (Lymphoprep; Axis-Shield, Norway) by density gradient centrifugation at $340 \times g$ for 15 minutes. After that, GCs were resuspended in red blood cell lysis buffer (Beyotime, China) for purification and washed with phosphate-buffered saline solution (PBS; Gibco, USA). Both the FF and the GCs were stored at -80°C until analysis.

Hormone and PGE2 Measurements

Serum hormone levels on days 2–5 of the menstrual cycle and trigger day of IVF, including FSH, LH, estradiol (E_2), P and testosterone (T) levels in serum, as well as the level of P and PGE2 in FF were measured using ELISA kit following the manufacturer's protocol (Beckman Coulter UniCel Dxl 800, USA).

Cell culture and treatment

Granulosa tumor-like cell line KGN (Bena culture collection, China) was used as *in vitro* cell model [15]. Cells were cultured in RPMI medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS, Biological Industries, Kibbutz Beit-Haemek, Israel), which were incubated at 37°C and 5% CO_2 in air. After 48 h of cell culture, the cells were pre-incubated in the serum-free medium for 12 h. Then the cells were cultured in serum-containing medium with progesterone dissolved with DMSO (1×10^{-6} , 1×10^{-5} , 1×10^{-4} , 1×10^{-3} , 1×10^{-2} nmol/L; sigma, USA) for 48 h or TNF- α (0–50 ng/mL; Sino Biological, China) for 10 h. Control cultures were exposed to medium added with the same volume of DMSO. Another group of cells were cultured in serum-containing medium with progesterone (1×10^{-4} nmol/L) for 0, 5, 15, 30, 60, 120 minutes respectively to explore the effects of progesterone on NF- κB signaling *in vitro*.

Quantitative Real-time PCR Analysis

The q-PCR analysis was carried out using SDS 2.3 software in 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) according to the standard protocol (see in detail in supplementary methods).

Western blot analysis

Proteins were extracted from human GCs or KGN cells, and lysed by RIPA lysis buffer (Cell Signaling Technology, USA) according to the manufacturer's protocol [16]. Western blot analysis were conducted according to the standard protocol (see in detail in supplementary methods).

Immunofluorescence

The cells pre-treated with progesterone (1×10^{-4} nmol/L) for 0, 5, 15, 30, 60, 120 minutes respectively and immunofluorescence analysis were conducted according to the standard protocol (see in detail in supplementary methods).

Chromatin Immunoprecipitation

In vivo crosslinking of proteins was performed by incubating KGN cells pretreated with TNF- α with 1% formaldehyde for 15 minutes at room temperature. After neutralization with glycine, cell lysates were vortexed with glass beads in lysis buffer containing 100 mM HEPES-KOH pH 7.5, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA, 140 mM NaCl, 2X cOmplete ULTRA Tablets (protease inhibitors, Roche, Switzerland), 1 mM phenylmethylsulphonyl fluoride, 50 mM NaF and 0.2 mM Na₃VO₄. Samples were then sonicated using a Covaris-S2 sonicator (Covaris, USA) to shear chromatin DNA into fragments of 100 to 500 bp. Immunoprecipitation of P65 bound to chromatin was performed using protein G Sepharose beads that were coupled to a rabbit monoclonal anti-p65 antibody (8242S, 1:100, Cell Signaling Technology, USA). Bead washes and elution, reverse cross-linking, and DNA precipitation were performed according to the Pierce Agarose ChIP Kit manual. Rabbit IgG was used as negative control. PCR was carried out later using different sets of primers spanning the *HPGD* promoter to verify the binding. The primer succeeded to detect a band in our ChIP-PCR assay is *HPGD*-F (-1333): 5'-TTATGGGGTGGGAGGGAGAC-3', *HPGD*-R (-1189): 5'-TTCTGTCACCTCAGCATGCA-3'.

Transcriptome analysis

Total RNA was extracted from cell by Trizol reagent (Invitrogen, USA). The RNA quality was checked by Bioanalyzer 2200 (Aligent, USA) and kept at -80°C. RNA with RIN >8.0 was used for rRNA depletion. The cDNA libraries of each pooled RNA sample for single-end sequencing were prepared using Ion Total RNA-Seq Kit v2.0 (Life Technologies, USA) according to the manufacturer's instructions. The cDNA libraries were processed for the Proton Sequencing process according to the commercially available protocols. Clean reads obtained from the raw reads by removing the adaptor sequences, reads with >5% ambiguous bases (noted as N) and low-quality reads containing more than 20 percent of bases with qualities of <13 were aligned to the human reference genome sequence GRCh38 (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/001/405/GCA_000001405.15_GRCh38) using the

MapSplice program (v2.1.6) [17]. Alternative Splicing Detector (ASD, available on <http://www.novelbio.com/asd/ASD.html>) was used as the tool to detect the differentially alternative splicing cases based on the bam file after mapping according to the P threshold ($P < 0.05$) [18]. HTseq was used to count gene and RPKM method was used to normalize gene expression level [19]. DEseq algorithm was used to identify the differentially expressed genes under the criteria that fold change > 1.5 and FDR < 0.05 [20]. Gene ontology (GO) analysis was performed to facilitate elucidating the biological implications of unique genes in the significant or representative profiles of the differentially expressed genes in the experiment [21]. GO annotations were integrated from NCBI (<http://www.ncbi.nlm.nih.gov/>), UniProt (<http://www.uniprot.org/>) and the Gene Ontology (<http://www.geneontology.org/>). Pathway analysis was used to find out the significant pathway of the differential genes according to KEGG database. Fisher's exact test were applied to identify the significant GO categories and pathways, and the false discovery rate (FDR) was used to correct the P -values. [22].

Statistical analysis

The results are presented as mean $\pm$ SEM. Quantitative variables were analyzed using Student's t-test for comparison between two groups. Statistical analysis was performed with Statistical Package for Social Sciences version 22.0 (SPSS 22.0). $P < 0.05$ was considered statistically significant.

Results

Clinical Characteristics of all Participants

A total of 50 patients with ovarian endometriosis and 50 controls were recruited in the present study. The baseline characteristics between endometriosis group and control group were comparable ($P > 0.05$), including age, body mass index (BMI), duration of infertility, mature, premature, abortion, existence, antral follicle count (AFC), total gonadotrophin dosage and retrieval oocytes (Table 1).

Table 1
Clinical Characteristics of all Participants.

	Control	Endometriosis	<i>P</i>
Number (n)	35	35	
Age (y, mean $\pm$ SD)	29.229 $\pm$ 2.680	29.314 $\pm$ 2.752	0.8954
Body mass index (kg/m ² , mean $\pm$ SD)	21.375 $\pm$ 2.039	20.660 $\pm$ 2.441	0.1875
Duration of infertility (y, mean $\pm$ SD)	3.886 $\pm$ 2.374	3.400 $\pm$ 2.376	0.3952
Mature (n, mean $\pm$ SD)	0.057 $\pm$ 0.236	0.086 $\pm$ 0.284	0.6483
Premature (n)	0	0	
Abortion (n, mean $\pm$ SD)	0.343 $\pm$ 0.684	0.200 $\pm$ 0.531	0.3324
Existence (n, mean $\pm$ SD)	0.057 $\pm$ 0.236	0.086 $\pm$ 0.284	0.6483
AFC (n, mean $\pm$ SD)	8.429 $\pm$ 3.791	7.943 $\pm$ 5.252	0.6587
Total Gn dogase (IU, mean $\pm$ SD)	2143.786 $\pm$ 778.794	1969.693 $\pm$ 876.802	0.3829
Basal FSH (IU/L, mean $\pm$ SD)	6.605 $\pm$ 1.725	7.453 $\pm$ 2.540	0.1931
Basal LH (IU/L, mean $\pm$ SD)	5.328 $\pm$ 2.284	5.210 $\pm$ 3.128	0.8578
Basal E2 (pmol/L, mean $\pm$ SD)	96.327 $\pm$ 26.497	109.410 $\pm$ 36.801	0.0924
Basal P (nmol/L, mean $\pm$ SD)	1.767 $\pm$ 0.428	2.124 $\pm$ 0.712*	0.0135
Basal T (nmol/L, mean $\pm$ SD)	0.549 $\pm$ 0.408	0.483 $\pm$ 0.458	0.5266
LH on trigger day (IU/L, mean $\pm$ SD)	2.444 $\pm$ 1.946	2.613 $\pm$ 2.495	0.7531
E2 on trigger day (nmol/L, mean $\pm$ SD)	11.051 $\pm$ 6.070	12.348 $\pm$ 11.762	0.5641
P on trigger day (nmol/L, mean $\pm$ SD)	2.643 $\pm$ 1.196	3.285 $\pm$ 1.395*	0.0472
Retrieval oocytes (n, mean $\pm$ SD)	12.029 $\pm$ 6.560	11.229 $\pm$ 8.117	0.6516
Values were given as mean $\pm$ standard deviations. p values were obtained using unpaired one tail t-test. <i>P</i> < .05 was considered statistically significant (n = 50). AMH: anti-mullerian hormone; AFC: antral follicle count; E2: estradiol; FSH: follicle-stimulating hormone; LH: luteinizing hormone; P: progesterone; T: testosterone. * <i>P</i> < .05.			

Elevated circulating progesterone in serum, PF and FF of endometriosis patients

To gain a better understanding of component changes in local endometriosis circulating, metabolomics analyses was conducted in PF and FF from both endometriosis group (n = 15) and control group (n = 15). Interestingly, we found that the only overlapping metabolic alteration between PF and FF from

endometriosis patients is the higher expression levels of progesterone (1.105 ± 0.019 vs 0.852 ± 0.025 , $P = 0.048$) (Fig. 1A).

To confirm metabolomics observations, the levels of reproductive hormone including FSH, LH, E2, T and P were further measured in both serum and FF from patients in endometriosis group and control group ($n = 35$). We found no differences in serum levels of FSH, LH, E2 and T on D2-5 (the second to the fifth day of menstrual cycle) and trigger day between the two groups ($P > 0.05$, respectively) (Table 1). However, compared with controls, higher levels of basal serum P (2.124 ± 0.020 nmol/L vs 1.767 ± 0.012 nmol/L, $P = 0.014$) (Fig. 1B) and higher serum levels of P on trigger day (3.285 ± 1.395 nmol/L vs 2.643 ± 1.196 nmol/L, $P = 0.047$) (Fig. 1C) were observed in endometriosis group, as well as significantly higher levels of P in FF (47.766 ± 9.612 nmol/L vs 39.519 ± 8.197 nmol/L, $P = 0.008$) (Fig. 1D).

Decreased expression of HPGD and COX-2 in GCs of endometriosis patients

To explore the effects of elevated P in FF on oocyte development and ovulation in endometriosis, we compared the genome-wide expression profiles of GCs from patients in both endometriosis group and control group ($n = 6$) via RNA sequencing. According to the sequence data, we identified 98 downregulated and 77 upregulated genes in GCs of endometriosis patients compared with controls (Supplementary table 1, Fig. 2A). *HPGD*, which play an important role in the expression of PGE2, is one of those down-regulated genes with statistical significance. RT q-PCR and Western-blot were carried out on a larger size of GCs samples from endometriosis patients ($n = 20$) and controls ($n = 20$) to confirm the differences in *HPGD* expression levels (Fig. 2B). COX-2, another key enzyme in the synthesis of PGE2, was also confirmed to be down-regulated in GCs samples from endometriosis patients (Fig. 2C).

PGE2 concentrations were further confirmed by ELISA in FF from both groups ($n = 35$) to explore the effects of decreased HPGD and COX2 on PGE2 levels. The results showed that PGE2 concentrations in FF from EMT group were higher than that in control group (400.663 ± 68.061 ng/L vs 330.791 ± 58.755 ng/L, $P < 0.001$) (Fig. 2D).

Repressed NF-κB signaling activity in GCs of endometriosis patients

As shown by gene ontology (GO) analysis, many down-regulated genes in GCs from endometriosis group were involved in NF-κB associated pathways including IL-1 mediated signaling, cytokine mediated signaling and Myd88-dependent toll-like receptor signaling pathways (Fig. 3A). The involved genes include *IL1R1* (-0.645 fold, $P = 0.028$), a receptor for IL-1, acting as an important pro-inflammatory cytokine in the NF-κB pathway; *MAP3K1* (-0.738 fold, $P = 0.100$), involved in one of the upstream pathways of NF-κB pathway ERK/MAPK signaling pathway, and *IRAK3* (-0.674 fold, $P = 0.030$), an important NF-κB signaling molecule (Supplementary Table 1). Similarly, RT q-PCR and Western blot were carried out on a larger size of GC samples from endometriosis patients ($n = 20$) and controls ($n = 20$) to confirm the differential expression of these molecules (Fig. 3B, 3C). Furthermore, decreased activation of IκBα and p65, two other important NF-κB signaling molecules were also confirmed in GCs from endometriosis patients via Western blot (Fig. 3C).

Effects of progesterone on HPGD and COX-2 expression and NF-κB signaling in GCs *in vitro*

Given the suppressed NF-κB signaling and downregulated *HPGD* and *COX-2* expression in GCs from endometriosis patients, we further explored whether these alterations were due to elevated P in FF. KGN cells were treated with P at different concentrations (1×10^{-6} , 1×10^{-5} , 1×10^{-4} , 1×10^{-3} , 1×10^{-2} nmol/L) *in vitro*. RT q-PCR and western blot revealed decreased expressions of IL1R1 and IRAK3, as well as HPGD and COX-2 (Fig. 4A, 4B, 4C) by P treatment in a dose dependent manner, which was most obvious at a concentration of 1×10^{-4} nmol/L in KGN cells.

The time-dependent effects of progesterone treatment (1×10^{-4} nmol/L) on NF-κB signaling in KGN cells was further investigated. Given that nuclear translocation of p65 is also a key step for NF-κB signaling transduction, the expression and localization of p65 were identified by Western blot on both cytoplasmic and nuclear protein and immunofluorescence in KGN cells respectively. The ratio of pIκBα/ IκBα in whole cell lysates were also determined by Western blot. The results revealed decreased NF-κB signaling activity by progesterone treatment *in vitro* in a time-dependent manner, which reached a plateau after 30 minutes of treatment, as manifested by the decreased ratio of pIκBα/ IκBα and reduced nucleus translocation of p65 (Fig. 4D, 4E, 4F).

Direct regulatory effects of NF-κB signaling on HPGD expression

KGN cells were exposed to TNF-α (50 ng/mL) to explore whether the activation of NF-κB signaling can up-regulate the expression of *HPGD*. The results showed that TNF-α not only obviously enhanced the transduction of NF-κB signaling by up-regulating pIκBα, p65, IL1R1 and IRAK3, but also up-regulated the expression of *HPGD* as expected (Fig. 5A, 5B). ChIP-PCR was conducted on KGN cells to explore whether there were binding elements for p65, an important downstream transcriptional factor, located at the promoter region of *HPGD*. We used PROMO (a virtual laboratory for the identification of putative transcription factor binding sites (TFBS) in DNA sequences from a species or groups of species of interest) and LASAGNA-Search 2.0 (Integrated Transcription Factor Binding Site Search and Visualization in a Browser) to search for the potential p65 binding sites in the promoter region of *HPGD*. After verified by ChIP-PCR, one potential p65 binding site was identified in the *HPGD* promoter (starting at 1261 bp upstream of the transcription start site, 5'-AGGAAAATTTCCAA - 3') (Fig. 5C, 5D).

Discussion

The present study showed a significantly elevated progesterone level in local circulating of endometriosis patients. Accordingly, NF-κB signaling was down-regulated in GCs derived from those patients, as manifested by decreased expression of IL1R1, IRAK3, reduced activation of IκBα and p65, compared with those of controls. *HPGD* and *COX-2* were further found to be down-regulated in endometriosis GCs, positively correlating with NF-κB signaling activation, and negatively associated with intrafollicular progesterone levels. Similarly, progesterone suppressed NF-κB signaling and reduced *HPGD* and *COX-2* expression *in vitro* in KGN cells. The NF-κB activator TNF-α, by contrast, up-regulated HPGD expression.

Furthermore, one potential binding element for p65 was identified in the promoter regions of *HPGD* by ChIP-PCR, suggesting a direct role of NF- κ B signaling in regulating *HPGD* expression.

Previous evidence showed that in women with moderate or severe endometriosis, some intrafollicular inflammatory cytokines were upregulated, unparallel with intrafollicular hormone concentrations [23, 24]. This may be due to the inflammatory microenvironment in women with endometriosis, impairing ovarian function and contributing to the reproductive dysfunction in endometriosis. However, whether some small molecular metabolites contribute to endometriosis-related ovulatory dysfunction is unknown. We collected both PF and FF from patients with ovarian endometriosis and control patients to explore the changes in the intrafollicular environment. Metabolic spectrum analysis showed significantly increased levels of progesterone in both FF and PF from endometriosis patients. It has been demonstrated that endometriotic stromal cells, which cause endometriotic lesions, were resistant to progesterone due to its deficiency in the expression of progesterone receptor (PR) [25]. Endometriotic tissues produce more progesterone and express significantly lower levels of PR compared with normal endometrium [26, 27]. The metabolic changes in FF and PF from patients with endometriosis could be a reflection of the component changes in the systemic or local circulation of endometriosis lesions in the ovary or peritoneal cavity [28]. We can assume that it is the progesterone resistance of the endometriotic tissues that cause the elevated levels of progesterone in serum, FF and PF.

Oocytes maturation is accompanied by GCs, the most common neighboring cells to FF in follicles. Therefore, we further conducted RNA sequencing in GCs from patients with ovarian endometriosis and healthy controls respectively to explore the alterations in RNA expression profile of GCs from endometriosis patients due to abnormal intrafollicular environment. The results revealed 98 down-regulated and 77 up-regulated genes in GCs from endometriosis group compared with controls. One of the most significantly altered signaling pathways was NF- κ B by GO analysis. Activated by inflammatory cytokines, NF- κ B was involved in cascade signal amplification of inflammation [29]. Constitutive activation and over-expression of NF- κ B were also observed in endometriotic stromal cells both *in vivo* and *in vitro* [30, 31]. Highly activated NF- κ B in the ectopic endometrium of women with endometriosis was confirmed to be closely related to local inflammation [30]. Ovulation has been considered a local inflammatory response. The mid-cycle surge of LH initiates a network of interconnected signaling cascades that lead to follicular rupture and oocyte release during ovulation. Many mediators of the LH-initiated signaling cascades are involved with inflammation, introducing the hypothesis that ovulation is similar to an inflammatory response. However, the role of NF- κ B signaling in endometriosis-related ovulatory dysfunction has not been explored yet.

In our study, decreased activation of NF- κ B was observed in GCs derived from patients with endometriosis. This seems to be contradictory with previous studies in endometrium. Progesterone is known to serve as an anti-inflammatory mediator [32]. Many recent studies have shown that progesterone acts through multiple mechanisms to inhibit NF- κ B [33]. As the direct lesion tissue of endometriosis, the endometrium may harbor specific epigenetic abnormalities altering expression of PR, leading to progesterone resistance [25] and activation of NF- κ B. Without those epigenetic abnormalities,

GCs may behave differently due to the local inflammation caused by the ectopic endometrial lesion. We assume that the elevated level of P in FF from patients with endometriosis may contribute to suppressing NF- κ B signaling in the neighboring GCs. However, the specific mechanism needs to be clarified.

COX-2 is a rate-limiting enzyme in the PGE2 compound[34], which has been reported as the key ovulatory prostaglandin [35, 36]. While HPGD acts as one of the primary catabolic enzymes of PGE2 and can modulate follicular PGE2 levels during the peri-ovulatory interval. Within the follicle, HPGD could maintain low intrafollicular PGE2 concentrations and lengthen the time between the ovulatory gonadotropin surge, thus facilitating the accumulation of PGE2 [35, 36]. Accumulation of PGE2 is widely thought to be a necessary and rate-limiting step in the process of ovulation in all mammalian species [37]. Duffy *et al* reported that exposure of GCs to progesterone decreased *HPGD* mRNA levels *in vitro* [35]. Similarly, down-regulation of *HPGD* and *COX2* in GCs from patients with endometriosis was also found in the present study. We assume that the decreased expression of *HPGD* and *COX-2* in GCs, due to the elevated levels of progesterone in FF in endometriosis, may synergistically contribute to the decreased PGE2 levels in FF of endometriosis patients.

To verify the above assumptions, we investigated whether progesterone-suppressed NF- κ B signaling pathway and down-regulated *HPGD* and *COX-2* expression *in vitro* in KGN cells, a granulosa tumor-like cell line. Our results revealed that progesterone significantly decreased the expression or suppressed the physiologic activation of NF- κ B signaling involved molecules including I κ B α and p65 [31, 38], which were also down-regulated in endometriosis GCs, *in vitro* in a dose-dependent manner. This observation suggests a role for progesterone in suppressing the NF- κ B signaling pathway in GCs. On the other hand, progesterone obviously decreased both the mRNA and protein expression of HPGD and COX-2 in KGN cells as well. The NF- κ B signaling pathway might participate in the regulation of HPGD expression by progesterone, as significantly increased HPGD expression was also observed after the induction of NF- κ B by TNF- α in KGN cells. Furthermore, we defined one potential binding element for p65 in the promoter region of *HPGD* by ChIP-PCR. These results suggest HPGD may be down-regulated by high levels of progesterone in FF through suppressing NF- κ B signaling in endometriosis.

Taken together, we found that patients with ovarian endometriosis presented with suppressed NF- κ B signaling pathway in GCs, which might be a negative factor to the timely regulation mode of PGE2 expression and ovulation. Intrafollicular progesterone might down-regulate *HPGD* and *COX-2* expression in GCs via suppressing the NF- κ B signaling pathway (Supplementary Fig. 1), while the regulation mechanism is to be investigated. Our study sheds light on the mechanism involved in the ovulatory dysfunction in patients with endometriosis and provide potential therapeutic targets for improvements of their ovulatory function. However, further studies are needed to explore mechanisms involved in the adverse effects of decreased PGE2 in FF on ovulation.

Conclusion

Higher FF progesterone may down-regulate *HPGD* and *COX2*, which play important roles in the process of ovulation by participating in the metabolism of PGE2, in GCs via suppressing NF-κB signaling and may lead to ovulatory dysfunction in endometriosis patients.

Declarations

Acknowledgements

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Authors' contributions

DZ and JYL conceived of the study, participated in its design and coordination and helped to draft the manuscript. JPC carried out the molecular studies, participated in the in vitro experiments and drafted the manuscript. YLQ and YFL carried out the ChIP-PCR experiments, JYM and FDN carried out the IHF experiments, RJZ and SWW is involved in revising the manuscript, YH, JL, XCW and BBW are involved in sample and clinical data collection, MXT and YYY help carrying out the WB experiments. YQW and BZ helped with the hormone assay.

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Availability of data and materials

The data underlying this article are available in the article and in its online supplementary material.

Ethics approval

This study was approved by the medical ethics committee of the Women's Hospital of Zhejiang University School of Medicine (Ethics Lot number 20170021).

Consent for publication

Not applicable.

Conflict of interest

The authors have none to declare.

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Figures

Figure 1

Elevated circulating progesterone in serum, PF and FF from endometriosis patients. (A) Comparison of progesterone expression levels in PF and FF from EMT group and control group (n=15) determined by metabolomics analyses. (B) Comparison of basal serum levels of progesterone on D2-D5 of menstrual cycle between EMT group and control group (n=35). (C) Comparison of serum levels of progesterone on the trigger day between EMT group and control group (n=35). (D) Comparison of progesterone concentration in FF between EMT group and control group (n=35). EMT: ovarian endometriosis. Each point in (B), (C) and (D) indicates the value of progesterone concentration in specific body fluid of each patient; bar showed standard error of mean in each group. $P < .05$ was considered statistically significant versus control group. * $P < .05$; ** $P < .01$.

Figure 2

Decreased COX-2 and HPGD expression in GCs from endometriosis patients. (A) Volcano plots of genes with differential expression in GCs from EMT group versus those from control group. The X axis represents the $\log_2$ of the fold change, and the Y axis represents the $-\log_{10}$ of the P-value from a student's t-test. The red points and blue points in the plot represent the up-regulated and down-regulated genes with statistical significance respectively (with a fold change ≥ 2 , and P-value < 0.05). (B) Differential transcriptional levels of COX-2 and HPGD mRNA in GCs from EMT group and control group were shown by qRT-PCR (n=20). (C) Differential expression levels of COX-2 and HPGD proteins in GCs from EMT group and control group (n=4) were shown by Western blot and the corresponding quantitative analysis. (D) Comparison of PGE2 concentration in FF between EMT group and control group (n=35). EMT: ovarian endometriosis. Each point in (B) and (D) indicates the value of the mRNA transcriptional

level in GCs or the PGE2 concentration in FF of each patient; Bar indicates standard error of mean in each group. $P < .05$ was considered statistically significant versus control group. * $P < .05$; ** $P < .01$.

Figure 3

Decreased expression of NF- κ B signaling pathway involved molecules in GCs from endometriosis patients. (A) GO analysis showed down-regulated signaling pathways in GCs from endometriosis patient by transcriptomics analysis. (B) Decreased transcriptional levels of ILIR1, IRAK3 and MAP3K1 in endometriosis patients compared with control patients were shown by qRT-PCR. Each point indicates specific mRNA transcriptional level in each patient; bar indicates standard error of mean in each group ($n=20$). (C) Decreased expressions of ILIR1, IRAK3, p65 and plkB α in endometriosis patients compared with control patients were shown by Western blot and the corresponding quantitative analysis. Bar indicates standard error of mean in each group ($n=4$). EMT: ovarian endometriosis. $P < .05$ was considered statistically significant versus control group. * $P < .05$; ** $P < .01$.

Figure 4

Progesterone regulate NF-κB signaling activity and expression of COX-2 and HPGD in KGN cells. Expression levels of ILIR1, IRAK3, MAP3K1 (A), COX-2 and HPGD (B) in KGN cells pretreated with progesterone at different concentrations (1×10^{-6} , 1×10^{-5} , 1×10^{-4} , 1×10^{-3} , 1×10^{-2} nmol/L) were shown by RT-qPCR (n=3). (C) Expression levels of COX-2, HPGD, IL1R1 and IRAK3 in KGN cells pretreated with progesterone at different concentrations (1×10^{-6} , 1×10^{-5} , 1×10^{-4} , 1×10^{-3} , 1×10^{-2} nmol/L) were

shown by Western blot. (D) Time-dependent effects of progesterone treatment on IκBα phosphorylation, p65 expression in KGN cells were shown by Western blot. Time-dependent effects of progesterone treatment on nucleus translocation of p65 in KGN cells were shown by Western blot conducted on cytoplasmic and nucleus proteins respectively (E) and immunofluorescence (n=3) (F), as well as the corresponding quantitative statistics. KGN cells were exposed to progesterone (1×10^{-4} nmol/L) for 0 min, 15 min, 30 min, 60 min and 120 min respectively. $P < .05$ was considered statistically significant versus 0 nmol/L or 0 min group (n=3). * $P < .05$; ** $P < .01$, *** $P < .001$.

Figure 5

Direct regulation effects of NF-κB signaling on HPGD expression. (A) Western blot showed increased expression levels of HPGD and NF-κB signaling associated molecules including IL1R1, IRAK3, p65, and plkBα in KGN cells pretreated by TNF-α (50 ng/mL). (B) q-PCR showed increased transcriptional of HPGD mRNA in KGN cells pretreated by TNF-α (50 ng/mL). $P < .05$ was considered statistically significant versus 0 ng/mL group. ** $P < .01$. (C) The prediction of potential p65 binding sites on HPGD promotor by lasagna-search 2.0, the red rectangle marks the one verified by ChIP-PCR further. (D) The schematic diagram of the positions of the potential p65 binding site on HPGD promotor and the information for the

corresponding primer used in ChIP-PCR assays and succeeding in detecting a specific band showing binding of p65 to the potential p65-binding site.

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