ORIGINAL ARTICLE: ENDOMETRIOSIS

# Investigating the impact of local inflammation on granulosa cells and follicular development in women with ovarian endometriosis

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**Objective:** To investigate the possible impact of local inflammation on granulosa cells (GCs) and follicular development in endometriosis patients.
**Design:** Prospective study with related paired design.
**Setting:** Reproductive medicine center.
**Patient(s):** A total of 80 endometriosis patients and 104 controls, with cultured GCs collected from control participants younger than 35 years.
**Intervention(s):** Tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and nuclear factor $\kappa$B (NF-$\kappa$B) inhibitor.
**Main Outcome Measure(s):** Intrafollicular concentrations of cytokines measured with ELISA, NF-$\kappa$B binding levels with electrophoretic mobility shift assay (EMSA), and telomerase activity (TA) with quantitative-telomeric repeat amplification protocol (Q-TRAP) assay, and protein and mRNA expression with Western blot and polymerase chain reaction analyses, respectively.
**Result(s):** Patients with endometriosis exhibited a statistically significantly lower antral follicle count ($11.48 \pm 8.11$ vs. $15.68 \pm 8.56$), lower number of retrieved oocytes ($8.28 \pm 6.69$ vs. $10.87 \pm 6.26$), and lower number of mature oocytes ($6.67 \pm 6.09$ vs. $8.53 \pm 5.69$). The GCs from endometriosis patients showed higher NF-$\kappa$B binding activity and increased expression of inhibitor of NF-$\kappa$B kinase subunit $\beta$ (IKK$\beta$, 2.743-fold) and NF-$\kappa$B inhibitor $\alpha$ (I$\kappa$B$\alpha$, 5.017-fold). Their NF-$\kappa$B p65 expression was negatively associated with mature oocytes ($b$NF-$\kappa$B$' = -0.304$, $R^2 = 0.195$, $R = 0.442$) but positively associated with intrafollicular TNF-$\alpha$ ($r = 0.37$); TA showed a negative relationship with NF-$\kappa$B binding levels ($r = -0.667$). Tumor necrosis factor-$\alpha$ induced expression of I$\kappa$B$\alpha$ (5.408-fold) and NF-$\kappa$B p65 (1.400-fold) but lowered human telomerase reverse transcriptase (hTERT) and TA levels (0.0009 vs. 0.5619) in cultured GCs. However, inhibiting NF-$\kappa$B obviously increased hTERT expression (1.988-fold).
**Conclusion(s):** Endometriosis showed activated NF-$\kappa$B pathways in GCs, which might negatively affect TA and oocyte quality. Intrafollicular TNF-$\alpha$ might down-regulate TA and hTERT via NF-$\kappa$B pathway, but further studies are required. (Fertil Steril® 2019;■: ■–■. ©2019 by American Society for Reproductive Medicine.)
**Key Words:** Endometriosis, hTERT, inflammatory cytokines, NF-$\kappa$ B, telomerase activity



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Endometriosis, a cause of subfertility, is found in almost 50% of infertile women (1). Some mechanisms have been documented to contribute to reproduction impairment in these women: poor oocyte quality and low-grade embryos were related to abnormal folliculogenesis (2), and decreased likelihood of fertilization was associated with increased levels of reactive oxygen species or local inflammation (3–5). This includes specifically, elevated levels of

cytokines in the follicular fluid of patients with endometriosis might account for the ovulatory dysfunction (6, 7).

Granulosa cells (GCs) play a large role in follicular development (8). In patients with endometriosis, abnormalities in GCs might impair oocyte maturation and lead to poor oocyte quality (9). Telomerase was identified as a biomarker of the growth potential of germ cells (10). Premature aging and reduced fecundity were obvious in telomerase-deficient mice (11). Our previous study found that telomerase activity of GCs (GTA) was positively correlated with in vitro fertilization (IVF) treatment outcomes (12). Healthy follicles presented with higher levels of telomerase activity (TA), while the reduction of GTA caused an increase in the number of atretic follicles (13). Patients with occult and biochemical primary ovarian insufficiency also showed diminished telomerase activity in their GCs (14, 15). Taken together, these results suggested that GTA might also be a biomarker of ovarian function.

Nuclear factor $\kappa$ B (NF-$\kappa$B) was the key point in inflammation. Activated by inflammatory cytokines, NF-$\kappa$B was involved in cascade signal amplification of inflammation or cellular events (16). Constitutive activation and overexpression of NF-$\kappa$B were also observed in endometriotic stromal cells under in vivo or in vitro conditions (17, 18). Inflammation might cause cell growth arrest by downregulating the expression of human telomerase reverse transcriptase (hTERT) and decreased TA levels (19), while activation of NF-$\kappa$B might also regulate TA (20). Therefore, in patients with endometriosis, NF-$\kappa$B might play an important role in the impact of local inflammation on GTA. GTA has been previously proven to be associated with follicular development; hence, these possible changes occurring in patients with endometriosis might cause impairment of oocyte maturation. However, no data about this issue have been reported.

In this study, we aimed to detect intrafollicular cytokines, NF-$\kappa$B pathway of GCs, GTA, and hTERT in patients with endometriosis and in cultured GCs to explore the association between local inflammation and follicular development. Furthermore, we aimed to identify the mechanism involved in the impairment of follicular growth in patients with endometriosis and provide potential therapeutic targets for improvements of their ovarian function.

## MATERIALS AND METHODS

### Study Population

A total of 80 patients with ovarian endometriosis and 104 controls, who underwent IVF or intracytoplasmic sperm injection treatment in the Reproductive Medicine Center of Sun Yat-Sen Memorial Hospital during February 2017 and December 2017, were enrolled in this study. Patients with ovarian endometriosis confirmed by laparoscopy with an interval of $<1$ year between the diagnosis and IVF were included. Patients with [1] regular menstrual cycle (23–35 days) (21), [2] basal follicle-stimulating hormone (FSH) level of $<10$ IU/L, [3] normal ovulation, and [4] tubal obstruction as an isolated cause of infertility comprised the control group. Patients aged over 45 years, or with pelvic inflammatory disease, with endocrine diseases, or with chromosome abnormality were excluded.

### Ethics Approval

This study was approved by the medical ethics committee of Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University. Written informed consent was obtained from all participants.

### IVF Protocol

Each participant underwent long protocol for ovarian stimulation. The participants were initially administered gonadotropin-releasing hormone agonist (GnRH-a) (Decapeptyl; Ferring GmbH) at 0.1 mg/day for pituitary desensitization in luteal phase before treatment with recombinant human FSH (Gonal-F; Merck Serono, Geneva, Switzerland) for gonadotropin stimulation. The dosage of FSH was based on the results of patients' vaginal ovarian ultrasound scans and serum estradiol ($E_2$) levels. Human chorionic gonadotropin (hCG; 6,000–10,000 IU; Lizhu Medical Company) was injected when three follicles were $\geq$16 mm, two follicles were $\geq$17 mm, or one follicle was $\geq$18 mm in diameter and serum $E_2$ levels reached or exceeded the level that corresponded to the size and number of follicles. Oocyte retrieval was scheduled 34–36 hours after hCG injection. Embryo transfer was performed 72 hours after the oocyte retrieval, with conventional luteal support. Five weeks after transfer, the presentation of gestational sac and fetal heart under the B-ultrasound indicated clinical pregnancy.

### Hormone Measurements

Basal hormone levels on days 2–4 of the menstrual cycle, including FSH, luteinizing hormone (LH), estradiol ($E_2$) and progesterone (P) levels, and peak $E_2$ levels on hCG day, were determined by Beckman Coulter UniCel DxI 800 and the associated reagents (Beckman Coulter). Serum antimüllerian hormone (AMH) levels were measured with enzyme-linked immunosorbent assay kit (ELISA; DSL Inc.).

### Follicular Fluid and GC Isolation

Follicular fluid for the concentration determination of the selected cytokines 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. We collected GCs samples on the oocyte retrieval day according to the protocol previously described elsewhere (12). After centrifugation for 5 minutes at 340 $\times$ $g$, GCs were gathered from the pellet with Ficoll solution (Lymphoprep; Axis-Shield) by density gradient centrifugation at 340 $\times$ $g$ for 15 minutes, and then resuspended in red blood cell lysis buffer (Beyotime) for purification. After they were washed with phosphate-buffered saline solution (PBS; Gibco), the cells were counted using a hemocytometer and then stored at $-80^{\circ}$C until analysis.

## Intrafollicular Cytokines Determination

Levels of interleukin 1$\beta$ (IL-1$\beta$), IL-6, and tumor necrosis factor $\alpha$ (TNF-$\alpha$) in follicular fluid were measured using ELISA kits (Invitrogen) according to the manufacturer's instructions. The intra-assay and interassay coefficients of variation for IL-1$\beta$ were 5.1% and 8.6%, respectively; for IL-6 were 3.4% and 5.2%, respectively; and for TNF-$\alpha$ were 6.0% and 9.3%, respectively. The lowest levels of sensitivity were 3.9 pg/mL for IL-1$\beta$, 1.56 pg/mL for IL-6, and 7.8 pg/mL for TNF-$\alpha$.

## Electrophoretic Mobility Shift Assay

Nuclear extraction of GCs was performed using a nuclear and cytoplasmic extraction kit (CWBio) following the manufacturer's protocols. The concentration of nuclear protein was measured using the bicinchoninic acid (BCA) protein assay kit (Beyotime) following the manufacturer's protocols. The binding level of NF-$\kappa$B in GCs was determined with an electrophoretic mobility shift assay (EMSA) as previously described elsewhere (22) using a chemiluminescent EMSA kit (Beyotime). The reaction mixture for each sample contained 2 $\mu$L of gel-shift binding buffer (5×), 5 $\mu$g of the nuclear fraction, and 0.5 $\mu$L of NF-$\kappa$B consensus oligo (5′-AGT TGA GGG GAC TTT CCC AGG C-3′, 3′-TCA ACT CCC CTG AAA GGG TCC G-5′) biotin-labeled probes (Beyotime), adjusted to 10 $\mu$L by nuclease-free water; the mixture then was incubated at room temperature for 30 minutes before we added 1 $\mu$L of loading buffer (10×). Separation of the mixtures was performed on a 4% polyacrylamide gel, which was then transferred to a nylon membrane and cross-linked in a ultraviolet light cross-linker for 10 minutes. The cross-linked membrane was incubated with the streptavidin-horseradish peroxidase conjugate (1:1,000) at room temperature for 4–5 hours and then washed before it was analyzed using a gel imaging system (Syngene).

## GTA Measurement

The quantitative-telomeric repeat amplification protocol (Q-TRAP) assay (23) was performed to detect GTA. We used a NP40 lysis buffer (Sigma-Aldrich) to lyse GCs. After being incubated for 30 minutes on ice, the lysate was centrifuged at 13,400 × $g$ for 30 minutes at 4°C. We then used a BCA protein assay kit (Beyotime) to measure the protein concentration.

During the Q-TRAP process, reaction mixtures included 12.5 $\mu$L 2× SYBR Prime Ex TaqII (Takara), 2 $\mu$L of extract, and 0.1 $\mu$g ACX primer (5′-GCGCGGCTTACCCTTACCCT-TACCCTAACC-3′), 0.1 $\mu$g TS primer (5′-AATCCGT CGAGCA-GAGTT-3′), and they were adjusted to 25 $\mu$L with nuclease-free water. After 30 minutes of incubation at 37°C, the polymerase chain reaction (PCR) was performed at 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 60 seconds. Telomerase-positive control 293T extracts and negative control 293T samples, which inactivated by incubating them for 10 minutes at 85°C, were included in each run. Positive samples were serially diluted to produce a standard curve ($R^2 = 0.99$), which was subsequently used to quantify the GTA. $\text{Relative GTA} = 10^{(\text{slope} \times Ct_{sample} + Y_{int})}$ (Supplemental Fig. 1, available online).

## Quantitative Real-time PCR Analysis

We measured RNA the expressions of inhibitor of NF-$\kappa$B kinase subunit $\beta$ (IKK$\beta$)/NF-$\kappa$B inhibitor $\alpha$ (I$\kappa$B$\alpha$)/nuclear factor $\kappa$B (NF-$\kappa$B) pathway and human telomerase reverse transcriptase (hTERT) by quantitative PCR (q-PCR). TRIzol reagent (Takara) was used to extract total RNA of GCs following the manufacturer's protocol. The RNA was then transformed to cDNA using the PrimeScript RT Master Mix System (Takara). The reaction mixtures for PCR included 5 $\mu$L SYBR Premix Ex TaqII (Takara), 2 $\mu$L cDNA samples, 0.4 $\mu$L forward primer, and 0.4 $\mu$L reverse primer (Supplemental Table 1, available online) and were adjusted to 10 $\mu$L using nuclease-free water. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was included in each run as an internal control.

The PCR process was performed on a Roche Light Cycler (Roche, Mannheim, Germany) at 95°C for 30 seconds, followed by 45 cycles at 95°C for 5 seconds and 60°C for 20 seconds. The relative RNA expression was quantified by normalizing the cycle threshold (Ct) values of target genes compared with GAPDH and calculated by the $2^{-\Delta\Delta Ct}$ method ($\Delta Ct = \text{Avg.}Ct_{sample} - \text{Avg.}Ct_{GAPDH}$, $\Delta\Delta Ct = \text{Avg.}\Delta Ct_{endometriosis} - \text{Avg.}\Delta Ct_{controls}$).

## Western Blot Analysis

Protein expression of phosphorylated IKK$\beta$ and I$\kappa$B$\alpha$, total IKK$\beta$ and I$\kappa$B$\alpha$, NF-$\kappa$B p65 and hTERT in GCs were analyzed by Western blot according to previously reported protocols (24). Cells were lysed with ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (CWBio). Protein concentration was then measured using BCA protein assay kit (Beyotime). Each sample (20 $\mu$g) was separated by 10% SDS-PAGE (Beyotime) according to the manufacturer's instructions, and blocked in 5% bovine serum albumin (2 g; Sigma-Aldrich) and 40 mL of Tris buffered saline buffer with Tween 20. They were probed with each primary antibody at 4°C overnight.

All antibodies used in this research were as follows: anti-IKK$\alpha/\beta$ (sc-7607; Santa Cruz Biotechnology), anti-p-IKK$\alpha/\beta$ (2681; Cell Signaling Technology), anti-I$\kappa$B$\alpha$ (4812; Cell Signaling Technology), anti-p-I$\kappa$B$\alpha$ (Ser 32) (9246; Cell Signaling Technology), anti-NF-$\kappa$B P65 (Sc372; Santa Cruz Biotechnology), anti-hTERT (sc-393013; Santa Cruz Biotechnology) and anti-$\beta$-actin (A5441; Sigma-Aldrich). We used $\beta$-actin in each run as an internal control. The protein expression was analyzed using a gel imaging system.

## Primary GCs Cultivation

To explore whether inflammatory cytokine (such as TNF-$\alpha$) regulated telomerase of GCs through NF-$\kappa$B pathway, we used primary GCs for in vitro treatment. Cells for cultivation were collected from women younger than 35 years who served as controls. After digestion and resuspension, the GCs were plated in six-well plates at a density of $5 \times 10^5$ cells per well and cultured overnight with 1:1 Dulbecco's modified Eagle's medium-Ham's F-12 media (Gibco) containing 10%

fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Gibco) in 5% $CO_2$ at 37°C in a constant temperature incubator (Sheldon). The medium was changed every other day until 80% to 90% confluent.

The cells were starved for 8 hours in serum-free medium before performing stimulation assays and then treated with TNF-$\alpha$ (0–50 ng/mL; Sino Biological). For the inhibition of NF-$\kappa$B, the GCs were treated with TNF-$\alpha$ (50 ng/mL) in the presence or absence of pyrrolidine dithiocarbamate (PDTC) (100 nM, NF-$\kappa$B inhibitor; Beyotime). The GCs were collected after 8 hours, and the expressions of NF-$\kappa$B pathway and hTERT as well as TA were measured according to the protocols described previously.

## Immunofluorescence

Immunofluorescence assay was conducted to confirm hTERT expression. After TNF-$\alpha$ treatment, the cells in the six-well plates were washed with PBS and incubated with 4% formaldehyde (HyClone) for 15 minutes at room temperature. After washing, the cells were permeabilized with 0.1%Triton (HyClone) before blocking with 5% bovine serum albumin. These GCs were incubated with anti-hTERT (1:100; sc-393013; Santa Cruz Biotechnology) antibody overnight at 4°C and then with secondary antibody in a 1:100 dilution for another 1 hour. Fluorescence microscopy was performed using the Nikon Eclipse TE 2000-S.

## Statistical Analysis

Statistical analysis was performed with Statistical Package for Social Sciences version 22.0 (SPSS 22.0; IBM). The Kolmogorov-Smirnov test was used to assess whether data were distributed normally. Quantitative variables were analyzed using Student's *t*-test or Mann-Whitney *U* test for comparison between two groups. Results were presented as mean ± standard deviation. Qualitative data like pregnancy rates, miscarriage rates, or ongoing pregnancy rates were compared with a chi-square test or Fisher's exact test. Correlation analysis was performed to evaluate the relationships between NF-$\kappa$B binding levels and GTA as well as the association between NF-$\kappa$B p65 expression and inflammatory cytokines levels, number of retrieval oocytes, number of mature oocytes, number of normal fertilized eggs, or number of normal cleavages. The general linear regression model was performed with correlation coefficient ($r$) and $R^2$ to analyze the correlations of NF-$\kappa$B p65 mRNA expression and the number of mature oocytes in patients with endometriosis. A paired *t*-test was performed for Western blot studies of P-I$\kappa$B$\alpha$/I$\kappa$B$\alpha$ ratio and P-NF-$\kappa$B/NF-$\kappa$B ratio in TNF-$\alpha$–treated and PDTC- treated cells versus control GCs cells. All experiments were repeated a minimum of three times. $P<.05$ was considered statistically significant.

# RESULTS

## Clinical Characteristics of all Participants

The ovarian endometriosis group consisted of patients with stage II and stage III endometriosis. The clinical characteristics of patients with endometriosis and controls are shown in Table 1. No differences were observed in age, body mass index (BMI), AMH, basal levels of FSH, $E_2$, and P, and antral follicle count (AFC) between the two groups. Compared with the controls, the patients with endometriosis had lower levels of basal LH (4.01 ± 3.93 IU/L vs. 4.42 ± 3.44 IU/L, $P=.029$), reduced AFC (11.48 ± 8.11 vs. 15.68 ± 8.56, $P<.001$), retrieved oocytes (8.28 ± 6.69 vs. 10.87 ± 6.26, $P<.001$), and mature oocytes (6.67 ± 6.09 vs. 8.53 ± 5.69, $P=.003$). Pregnancy outcomes did not differ between the two groups ($P>.05$).

## Associations Between Intrafollicular Cytokines and NF-$\kappa$B p65 Expression

Higher levels of IL-1$\beta$ (2.270 ± 1.673 pg/mL vs. 2.027 ± 1.032 pg/mL), IL-6 (15.667 ± 36.02 pg/mL vs. 7.793 ± 10.676 pg/mL), and TNF-$\alpha$ (10.086 ± 10.978 pg/mL vs. 7.721 ± 4.034 pg/mL) were found in the follicular fluid of endometriosis, but these results were not statistically significant ($P>.05$).

The mRNA expression of IKK$\beta$ (2.743-fold, $P=.004$) and I$\kappa$B$\alpha$ (5.017-fold, $P=.004$) established in GCs from patients with ovarian endometriosis were statistically significantly increased (Fig. 1A). Phosphorylated levels of IKK$\beta$ ($P=.04$) and I$\kappa$B$\alpha$ ($P=.019$) were also increased in the endometriosis group (Fig. 1B). There was an increase in the NF-$\kappa$B p65 protein expression in GCs from endometriosis patients ($P=.017$) (Fig. 2A). No obvious difference was observed in NF-$\kappa$B p65 mRNA expression between the two groups (1.014-fold, $P>.05$). The correlation analysis revealed that NF-$\kappa$B p65 mRNA expression was positively associated with the intrafollicular TNF-$\alpha$ levels in patients with endometriosis ($R^2 = 0.14$, correlation coefficient [$r$] = 0.37, $P=.031$).

## Associations Between NF-$\kappa$B Binding Activity and GTA

Patients with endometriosis exhibited obviously increased levels of NF-$\kappa$B binding activity in GCs compared with controls ($P=.012$) (Fig. 2B). The mRNA expression of hTERT was higher in patients with endometriosis (1.817-fold, $P=.005$). By contrast, the protein expression of hTERT was decreased in women with ovarian endometriosis, but this finding was not statistically significant ($P=.055$) (Fig. 2C). Also, GTA showed a downward trend in these patients compared with controls, but this finding was not statistically significant (0.343 vs. 0.465, $P=.161$) (Fig. 2D). In GCs from patients with endometriosis, the correlation analysis revealed that TA was negatively associated with NF-$\kappa$B binding levels ($R^2 = 0.44$, $r = -0.667$, $P=.0498$).

## Association Between NF-$\kappa$B p65 Expression, GTA, and Mature Oocytes

Among patients with endometriosis, GTA was positively associated with the number of mature oocytes ($R^2 = 0.06$, $r = 0.237$, $P=.033$); the mRNA expression of NF-$\kappa$B p65 in GCs was negatively correlated with the number of retrieval oocytes ($R^2 = 0.11$, $r = -0.339$, $P=.003$), mature oocytes ($R^2 = 0.16$, $r = -0.403$, $P<.001$), normal fertilized eggs ($R^2 = 0.14$, $r = -0.373$, $P=.001$), normal cleavage of fertilized

**TABLE 1**

**Clinical characteristics of patients with endometriosis and controls.**

| Characteristic | Endometriosis | Controls | *P* value |
|---|---|---|---|
| N | 80 | 104 | |
| Age (y) | 34.18 ± 4.52 | 33.95 ± 5.43 | .649 |
| BMI (kg/m$^2$) | 21.25 ± 2.87 | 20.61 ± 3.04 | .458 |
| AMH (ng/mL) | 2.86 ± 3.30 | 3.65 ± 3.90 | .087 |
| CA-125 (IU/mL) | 45.58 ± 85.63 | 24.53 ± 25.02 | .160 |
| Basal FSH (IU/L) | 8.76 ± 3.80 | 8.10 ± 2.37 | .139 |
| Basal LH (IU/L) | 4.01 ± 3.93 | 4.42 ± 3.44 | .029[a] |
| Basal $E_2$ (ng/L) | 59.49 ± 73.32 | 51.94 ± 61.99 | .099 |
| Basal P (ng/L) | 1.20 ± 2.27 | 0.85 ± 0.59 | 1.000 |
| AFC | 11.48 ± 8.11 | 15.68 ± 8.56 | <.001[a] |
| Total Gn dosage (IU) | 2,214.82 ± 1,098.72 | 2,171.74 ± 882.90 | .575 |
| Peak $E_2$ (pg/mL) | 1,881.95 ± 1,190.14 | 2,242.76 ± 1,040.59 | .005 |
| Retrieval oocytes | 8.28 ± 6.69 | 10.87 ± 6.26 | <.001[a] |
| Mature oocytes | 6.67 ± 6.09 | 8.53 ± 5.69 | .003[a] |
| Normal fertilized eggs | 4.02 ± 4.63 | 6.40 ± 4.04 | .001 |
| Normal cleavage no. of fertilized eggs | 4.96 ± 4.61 | 6.20 ± 3.93 | .002 |
| Available embryos | 2.75 ± 2.46 | 3.24 ± 2.99 | .168 |
| Rate of good-quality embryos | 57.71 ± 53.44 | 60.09 ± 55.45 | .845 |
| Frozen embryos | 1.81 ± 2.18 | 2.01 ± 2.81 | .907 |
| Clinical pregnancy, n (%) | 36 (45.0) | 41 (43.6) | .855 |
| Miscarriage, n (%) | 4 (11.1) | 5 (12.2) | 1.000 |
| Ongoing pregnancy, n (%) | 32 (40.0) | 36 (38.7) | .862 |

*Note:* Values were given as number or mean ± standard deviations or numbers (percentages). *P* values were obtained using either Mann-Whitney *U* test or *t*-test or chi-square test. $P<.05$ was considered statistically significant. Rate of good-quality embryos refers to the ratio between the number of good-quality embryos and the number of embryos available for transfer. AFC = antral follicle count; AMH = antimüllerian hormone; BMI = body mass index; $E_2$ = estradiol; FSH = follicle-stimulating hormone; Gn = gonadotropin; LH = luteinizing hormone; P = progesterone.

[a] $P<.05$.

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eggs ($R^2 = 0.12$, $r = -0.352$, $P=.002$), and embryos available for transfer ($R^2 = 0.07$, $r = -0.27$, $P=.021$) (Fig. 2E). In the control group, no association was observed between NF-κB mRNA expression and the number of retrieval oocytes, mature oocytes, or available embryos ($P>.05$).

To analyze the factors associated with the number of mature oocytes in patients with endometriosis, the following factors were included in the multiple linear regression analysis: NF-κB p65 mRNA expression, GTA, AFC, age, BMI, basal FSH and $E_2$ levels, and total gonadotropin dosage. The results revealed that in patients with endometriosis, AFC, and NF-κB p65 expression of GCs were closely associated with the number of mature oocytes: Mature oocytes = 9.333 + 0.208 × (AFC) − 3.743 × (NF-κB p65 mRNA expression), $b$NF-κB′ = −0.304, $R^2 = 0.195$, $R = 0.442$, $P=.018$.

## Effect of TNF-α on NF-κB Signaling Pathway in Primary GCs

In cultured primary GCs, the exposure of TNF-α in the concentration up to 50 ng/mL obviously enhanced the mRNA expression of both IκBα (7.4-fold, $P<.001$) and NF-κB p65 (1.4-fold, $P=.031$) (Fig. 3A). Treated with higher concentrations of TNF-α, the phosphorylated levels of IκBα ($P=.26$) and NF-κB p65 ($P=.04$) protein were also elevated in primary GCs (Fig. 3B).

## Effect of TNF-α on hTERT and TA in Primary GCs

Tumor necrosis factor α obviously weakened hTERT protein expression (Fig. 3B) and immunofluorescent staining (Fig. 3C), and statistically significantly decreased TA levels of GCs under in vitro cultivation (0.5619 vs. 0.0009, $P=.047$) (Fig. 3D). The mRNA expression of hTERT did not statistically significantly change after TNF-α treatment ($P>.05$) (Fig. 3A).

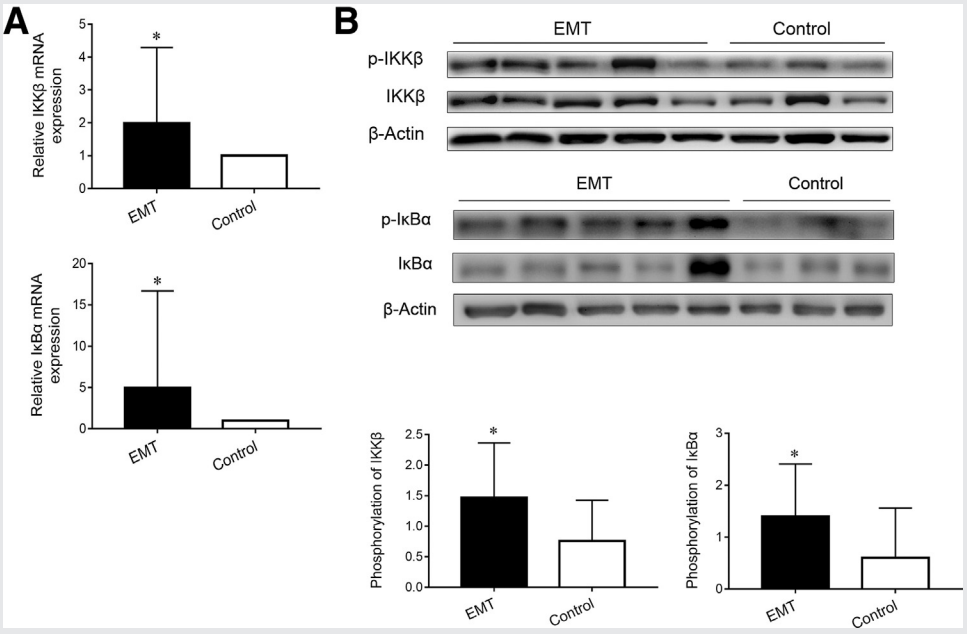
## Effect of NF-κB Inhibitor on hTERT and TA in Primary GCs

The activity of NF-κB activity was inhibited by PDTC through down-regulating phosphorylated IκBα levels. In cultured GCs, the addition of PDTC obviously decreased the phosphorylated protein levels of IκBα ($P=.02$) and NF-κB p65 ($P=.01$) as elevated by TNF-α (Fig. 3E). Conversely, PDTC statistically significantly increased the mRNA expression of hTERT (1.98-fold) ($P=.007$) (Fig. 3F), while GTA remained unchanged ($P>.05$) (Fig. 3G).

# DISCUSSION

This study showed that in GCs derived from patients with ovarian endometriosis the IKKβ and IκBα expression and NF-κB binding levels were statistically significantly increased compared with those of healthy controls. In patients with endometriosis, GTA was negatively correlated with the levels of NF-κB binding, and NF-κB mRNA expression was positively associated with intrafollicular TNF-α levels but negatively associated with the number of mature oocytes. Moreover, in cultured primary GCs, TNF-α obviously activated NF-κB signaling pathway but reduced GTA levels and hTERT expression; after we added the NF-κB inhibitor

## FIGURE 1




Comparison of IKK$\beta$/I$\kappa$B$\alpha$/NF-$\kappa$B pathway expression of GCs between the endometriosis group and controls. EMT = endometriosis. (**A**) Comparison of IKK$\beta$ and I$\kappa$B$\alpha$ mRNA expression between patients with endometriosis and controls. Each bar and column indicates standard deviation and mean, respectively. *$P$<.05 was considered statistically significant ($P$IKK$\beta$=.004, $P$I$\kappa$B$\alpha$=.004). (**B**) Comparison of the protein expression of IKK$\beta$/I$\kappa$B$\alpha$/NF-$\kappa$B pathway between the two groups. P-IKK$\beta$ and p-I$\kappa$B$\alpha$ = phosphorylated IKK$\beta$ and I$\kappa$B$\alpha$, respectively. Increased phosphorylated levels of IKK$\beta$ ($P$=.040) and I$\kappa$B$\alpha$ ($P$=.019) were observed in GCs from patients with endometriosis.

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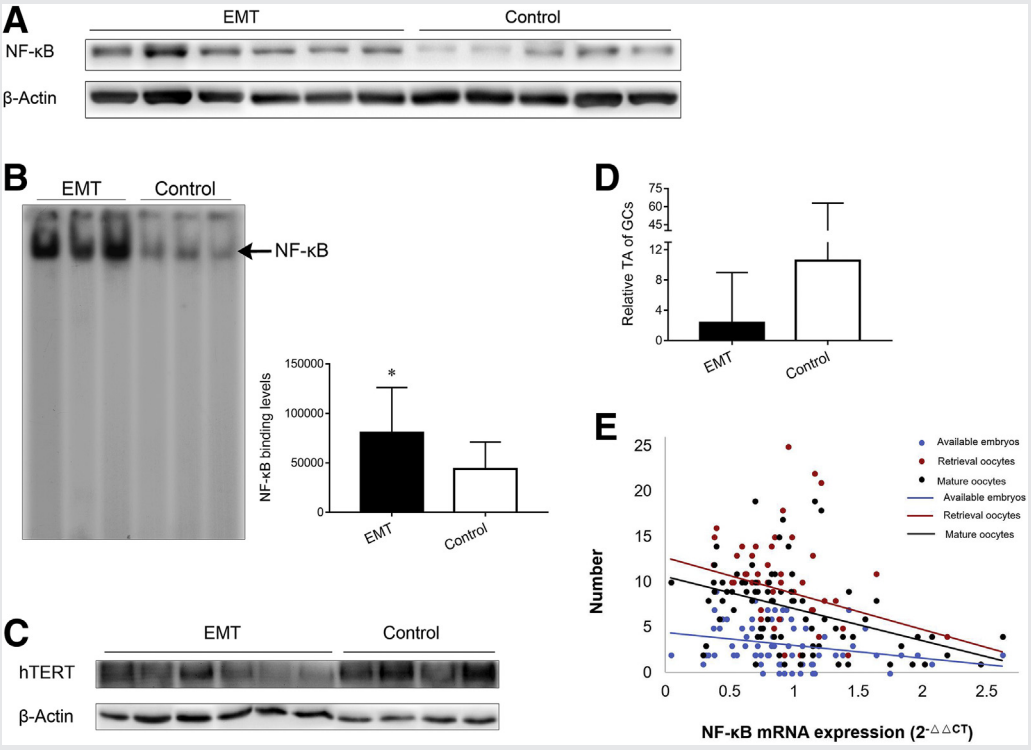
PDTC, hTERT expression was statistically significantly up-regulated.

Nuclear factor $\kappa$B was the nodal point of a primary inflammation stimulated signaling pathway (25). Highly activated NF-$\kappa$B in the eutopic endometrium of women with endometriosis was confirmed to be closely related with local inflammation (17). Previous evidence showed that elevated intrafollicular cytokines in patients with ovarian endometriosis (6) might impair their ovarian function and lead to poor oocyte quality. Oocytes mature in follicles surrounded with GCs, so we collected GCs from patients with ovarian endometriosis and healthy controls to examine whether NF-$\kappa$B of GCs might be associated with the abnormal intrafollicular environment and oocyte maturation of endometriosis. Our results showed that NF-$\kappa$B binding activity was detectable in GCs and indicated a marked increase in patients with ovarian endometriosis. These results suggested that the activation of NF-$\kappa$B was higher in GCs of patients with endometriosis. Moreover, in these patients, NF-$\kappa$B p65 mRNA expression was positively associated with intrafollicular TNF-$\alpha$ levels. Inflammatory cytokines such as TNF-$\alpha$ activated NF-$\kappa$B by inducing phosphorylation of IKK$\beta$ and I$\kappa$B$\alpha$ (26). In our study, the physiologic activation and mRNA expression of IKK$\beta$ and I$\kappa$B$\alpha$ were statistically significantly higher in patients with ovarian endometriosis, indicating that these patients had abnormal activation of NF-$\kappa$B signaling pathway in GCs.

In GCs from patients with primary ovarian insufficiency, TA is significantly decreased (14). Our previous study demonstrated that GTA was also significant for predicting IVF outcomes (27). In our study, GTA showed a positive relationship with the number of mature oocytes in patients with endometriosis. Compared with the controls, the patients with ovarian endometriosis presented with less mature oocytes and a downward trend of GTA. These results suggest that GTA might have a close relationship with oocyte quality of patients with endometriosis. Our results also showed that GTA was negatively associated with NF-$\kappa$B–DNA binding activity in these patients. Moreover, activated NF-$\kappa$B was involved in the regulation of cell growth and apoptosis (28). Relationships between GTA and NF-$\kappa$B might be explained as follows. First, as one of the transcription factors, NF-$\kappa$B might bind to the transcriptional regulatory region of hTERT promoter to directly regulate TA levels (20). Nuclear factor $\kappa$B might also affect the expression of other transcription factors for hTERT (29), playing an indirect role in TA regulation. In our study, hTERT mRNA expression of patients with endometriosis was much higher than that of healthy controls, but the protein expression showed an opposite trend. We supposed that transcriptional regulation possibly occurred in GCs of patients with ovarian endometriosis, as their GTA showed a downward trend.

Correlation analysis and multiple linear regression analysis revealed that NF-$\kappa$B p65 of GCs was a negative factor in terms of oocyte maturation in those with ovarian endometriosis. This relationship between NF-$\kappa$B and oocyte quality can be explained as follows. First, we found a positive relationship between NF-$\kappa$B p65 expression and TNF-$\alpha$ levels.

## FIGURE 2




Comparison of NF-κB of GCs and GTA between the two groups. (**A**) Comparison of NF-κB protein expression ($P$=.017). (**B**) Levels of NF-κB-DNA binding in patients with endometriosis and controls. Column chart presents the quantitative comparison of NF-κB-DNA binding levels. Each bar and column indicates standard deviation and mean, respectively. *$P$=.012. (**C**) Protein expression of hTERT in GCs from patients with endometriosis showed a downward trend compared with that from controls ($P$=.055). (**D**) The right column chart presents the comparison of GTA between these two groups. Each bar and column indicates standard deviation and mean, respectively. The GTA of patients with endometriosis was lower than that of controls but was not statistically significant ($P$=.161). (**E**) Distribution of the number of retrieval oocytes, mature oocytes, and embryos available for transfer according to mRNA expression levels of NF-κB p65 in endometriosis GCs. Linear regression lines indicate retrieval oocytes (red line, $r = -0.339$, $P$=.003), mature oocytes (black line, $r = -0.403$, $P$<.001), and available embryos (blue line, $r = -0.27$, $P$=.021), respectively. Red circles indicate the number of retrieval oocytes, black circles indicate the number of mature oocytes, and blue circles indicate the number of available embryo number.

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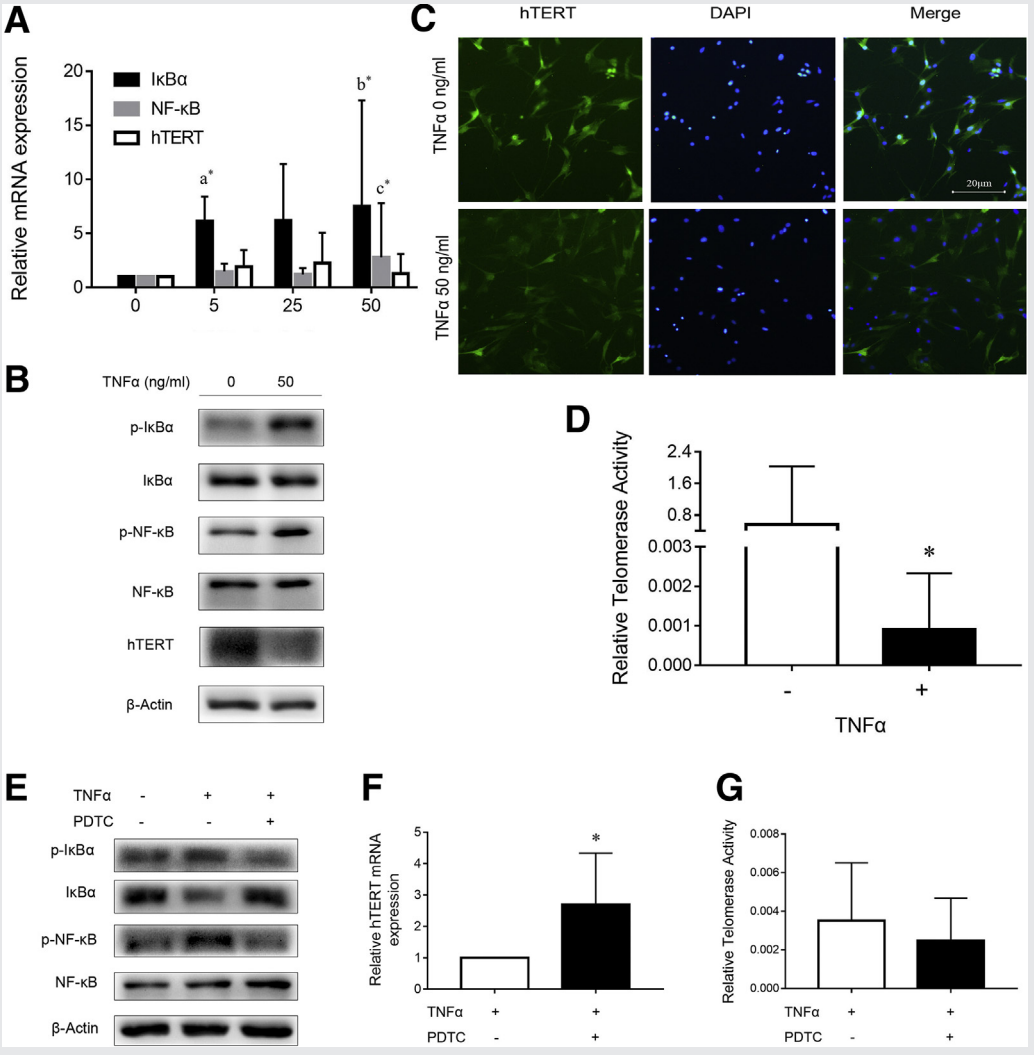
As a master regulator of inflammation induced by TNF-α, NF-κB activation stimulated the expression and secretion of IL-6 and IL-1β (30). Cultured GCs derived from patients with endometriosis showed a statistically significant increase in the secretion of IL-6 (31), which would attenuate aromatase activity and estrogen biosynthesis (32). These changes might be associated with oocyte maturation. Besides, NF-κB binding levels were statistically significantly increased in these patients, but there was a negative association between NF-κB binding activity and GTA. Furthermore, decreased GTA would suppress follicular development and induce follicular atresia (13). Diminished GTA and shortened GTL were closely associated with ovarian insufficiency (15). These findings might also explain why NF-κB mRNA expression was negatively associated with the number of available embryos.

The TNF-α levels are statistically significantly increased in the peritoneal and follicular fluid of patients with endometriosis (33). Produced by macrophages or GCs (7), TNF-α is central to the endometriotic disease process and contributed to local inflammation in these patients (34). As described previously, we observed a positive relationship between the NF-κB p65 expression of GCs and intrafollicular TNF-α levels among women with ovarian endometriosis. Patients with moderate and severe endometriosis had increased GC apoptosis (35); in our study, a negative relationship was found between GTA and NF-κB binding levels in patients with endometriosis. Based on these findings, we used primary GCs collected from control women to investigate whether TNF-α induced NF-κB signaling pathway and down-regulated TA. Our experimental results revealed that TNF-α statistically significantly increased mRNA expression and physiologic activation of IκBα and NF-κB p65 in cultured GCs, suggesting that TNF-α might activate the NF-κB signaling pathway of GCs. On the contrary, TNF-α obviously decreased TA levels and hTERT expression in these cells, indicating that GCs might not benefit from elevated TNF-α levels in local environment.

The NF-κB signaling pathway might participate in TNF-α's impairment of hTERT, as statistically significantly increased hTERT expression was observed after the inhibition

## FIGURE 3




The effect of TNF-$\alpha$ and PDTC on the NF-$\kappa$B signaling pathway, hTERT expression, and TA levels in primary GCs. (**A**) TNF-$\alpha$ at concentration of 50 ng/mL statistically significantly increased mRNA expression of I$\kappa$B$\alpha$ and NF-$\kappa$B p65. [a,b,c]Statistically significant compared with nontreated GCs ([a]*$P$<.001, [b]*$P$<.001, [c]*$P$=.031). (**B**) Increasing levels of TNF-$\alpha$ statistically significantly induced phosphorylation of I$\kappa$B$\alpha$ ($P$=.26) and NF-$\kappa$B p65 ($P$=.04) protein expression. (**C**) The effect of TNF-$\alpha$ on immunofluorescent staining of hTERT in cultured GCs. Staining of hTERT (green) and staining of DAPI (blue) are shown (scale bar, 20 $\mu$m). (**D**) TNF-$\alpha$ (50 ng/mL) statistically significantly diminished TA levels in primary GCs (*$P$=.047). The minus sign represents equal volume of PBS, and the plus sign represents 50 ng/mL TNF-$\alpha$. Each bar and column in the column chart represents standard deviation and mean, respectively. (**E**) PDTC decreased phosphorylation of I$\kappa$B$\alpha$ ($P$=.02) and NF-$\kappa$B p65 ($P$=.01) as elevated by TNF-$\alpha$. (**F**) The inhibition of the NF-$\kappa$B pathway statistically significantly increased mRNA expression of hTERT (*$P$=.007). (**G**) GTA did not statistically significantly change ($P$>.05) after treatment with PDTC. Plus sign represents 50 ng/mL for TNF-$\alpha$ or 100 nM for PDTC, and minus sign indicates equal volume of PBS. Each bar and column indicates standard deviation and mean, respectively.

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of NF-$\kappa$B in cultured GCs. We did not observe obvious changes in GTA after inhibiting NF-$\kappa$B. These findings could be caused by other factors. Changes in hTERT expression might occur earlier than TA (36); therefore, an obvious increase in hTERT expression was only observed after an 8 hours of cultivation of GCs with PDTC in our study. Some researchers also have recommended the pretreatment of inhibitor or the use of small interfering RNA to completely block the signaling pathway (37). Cultivation of primary GCs might have its limitations in cultured time; only the binding activity and the expression of protein and mRNA of NF-$\kappa$B p65 were detected in this research.

Patients with ovarian endometriosis presented with activated NF-$\kappa$B signaling pathway in GCs, which might be a negative factor to GTA and oocyte quality. Intrafollicular TNF-$\alpha$ might down-regulate TA and hTERT of GCs via the NF-$\kappa$B signaling pathway, but further studies are required to confirm the regulation mechanism.

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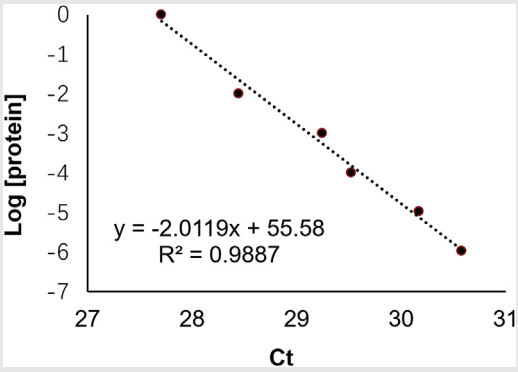
## SUPPLEMENTAL FIGURE 1




Detection of the standard curve for Q-TRAP. The equation was calculated by plotting log [protein] (protein means the protein concentration) against the Ct values of the positive control. The relative TA of each sample would be calculated with the *Y*-intercept and the slope values using the following equation: $\mathrm{GTA} = 10^{\left(\mathrm{Slope} \times Ct_{sample} + Y_{int}\right)}$.

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