Role of NF-κB pathway in the transition of mouse secondary follicles to antral follicles

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Running title:*NF-κB is involved in follicular development*

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Abstract

NF-kappaB (NF-κB) signaling is involved in regulating a great number of normal and abnormal cellular events. However, little is known about its role in ovarian follicular development. In this study, we found NF-κB signaling is activated during the transition from secondary to antral follicles. We generated active NF-κB mice and found that antral follicular numbers were higher than wild-types ovaries. Activation of NF-κB signaling could enhance granulosa cell proliferation and regress granulosa cell apoptosis of mouse ovarian follicles. Higher FSHR and LHCGR expressions were observed in active NF-κB ovaries compared to wild-type. Furthermore, we confirmed that NF-κB signaling was indeed involved in the granulosa cell viability and proliferation through FSHR using COV434 cell line. This is the first experimental evidence that NF-κB signaling is implicated in the control of follicular development through FSHR and its corresponding target molecules, which might be achieved by targeting proliferation and apoptosis in follicular granulosa cells.

Key words: NF-κB signaling, follicular development, granulosa cells, cell proliferation and apoptosis.

Introduction

The ovarian follicles are the fundamental functional unit of the ovary, and they are morphologically composed of an oocyte surrounded by granulosa and theca cells, which protect and support the development of the oocytes. Ovarian follicle maturation proceeds through primordial, primary, secondary and mature antral follicular stages. At birth, the ovary contains approximately one million hibernating primordial follicles and some of them become activated to go through folliculogenesis during puberty. The various developmental stages that the activated primordial follicles pass through subsequent to follicular development during folliculogenesis are also shared by many animal species. The numbers of primary follicles, which derive from a large number of primordial follicles, decreases when they develop to secondary and antral follicles under the appropriate hormonal environment. Most follicles normally degenerate to atretic follicles, and this can occur at all stages of follicular development (McGee & Hsueh, 2000).

Ovarian follicle development is precisely regulated by a sequence of autocrine and paracrine factors. Additionally, it relies on the input from endocrine hormones including pituitary and ovarian hormones. The balance of these hormones is especially vital since it determines whether a developing follicle becomes maturated or undergoes atresia (Bertoldo, Bernard, Duffard, Mermillod, & Locatelli, 2013; Matsuda, Inoue, Manabe, & Ohkura, 2012; Raju et al., 2013). Amongst these hormones, follicle-stimulating hormone (FSH) is undoubtedly the most important, because it plays a role in both the survival of early antral-staged follicles and the growth, activation and differentiation of prenatal follicles (Fauser, 1994; Hsueh, McGee, Hayashi, & Hsu, 2000). FSH-dominated the exponential growth of ovarian follicles acts principally as a consequence of the proliferation of granulosa cells. Beyond that, activin also plays a very important role on regulating the proliferation and differentiation of granulosa cells, but it is indispensable for the removal of FOXO1-dependent repression and positive signaling by Smad2/3 (Park et al., 2005). Accumulating evidence indicates that the death of follicular granulosa cells is partly responsible for causing follicular atresia (Liu, Yue, Ma, Sun, & Tan, 2003; Murdoch,

1995). Interfering with steroidogenesis and the dexamethasone exposure could lead to apoptosis of granulosa cells, which in turn triggers follicular atresia. In contrast, insulin-like growth factor (IGF) could restrict follicular atresia through preventing apoptosis in granulosa cells as induced by dexamethasone (deMoura, Chamoun, Resnick, & Adashi, 2000).The cellular and molecular mechanisms underlying the developmental fate of ovarian follicles is not entirely understood (Yu et al., 2004), therefore, more elaborative studies on regulating follicular development are necessary to elucidate the underlying molecular biological mechanisms in response to FSH and activin.

Nuclear factor-κB (NF-κB) was identified as a regulator of expression of the κB light chain in B cells thirty years ago (Hayden & Ghosh, 2008; Sen & Baltimore, 1986). But, NF-κB has being intensively studied since a variety of internal and external stimuli could activate the transcription factors, which also regulate numerous crucial gene expressions in a multiple organisms during physiological and pathological events. For a long time, it has been considered that the NF-κB is the only immunologically relevant signaling pathway since it has been found to play an important and indispensable role on regulating the expression of inducers and effectors in the unreserved networks that define responses to pathogens (Razani, Reichardt, & Cheng, 2011). However, the biological forces of NF-κB signaling reach extensively to transcriptional regulation beyond the boundaries of the immune response, acting widely to impact on gene expression events that are involved in cell survival, differentiation and proliferation. NF-κB family of transcription factors is composed of five members including p50, p52, p65 (RelA), c-Rel, and RelB. Activation of NF-κB proceeds through the liberation of NF-κB dimers from inactive state, in which NF-κB dimers are associated with one of three IκB proteins. The released NF-κB undergoes thetranslocation to nucleus, where the transcription of target genes is promoted via its binding to specific DNA sequences. A variety of stimuli including both endogenous and exogenous stresses could activate NF-κB signaling (Chen & Greene, 2004). Meanwhile, the correlations between NF-κB signaling and other physiological signaling pathways are still obscure although new

studies continue to provide more evidence (Adler et al., 2007).More interestingly, despite it has been known that ovarian follicular atresia is coupled with granulosa cell apoptosis, the regulators initiating granulosa cell apoptosis have not been fully addressed. There was a report that NF-κB signaling was deemed to be one of vital genes controlling granulosa cell apoptosis (Valdez & Turzillo, 2005). But, more precise experimental evidence is certainly required to reveal the interplaying and underlying mechanism. In this study, we investigated whether NF-κB signaling was involved in regulating follicular development and atresia through its effect on granulosa cell survival using activated NF-κB transgenic mice. We systematically examined the development of the ovarian follicles in activated NF-κB transgenic mice and especially focused on the correlation between antral follicular development and granulosa cell growth and death.

Materials and Methods

Mice

Knockin (NF-κB1^{C59S}) mice were obtained from Modern Animal Research Center of Nanjing University. Exon 6 of the mouse NF-κB1（p50）gene, codon TGT for Cys-59 was mutated to TCA encoding Ser by means of site-directed mutagenesis. p50 with this substitution retained a maximum DNA-binding activity (Mitomo et al., 1994; Toledano, Ghosh, Trinh, & Leonard, 1993). This means that the NF-κB signal is activated to some extent. A PGK-neo cassette was inserted in an intron near the mutation point as a selective marker. Standard cloning techniques were used to construct targeting vectors. The fragment containing the 5kb 5' arm, mutation point, PGK-neo and 5kb 3'arm. The targeting vector was linearized and transferred to the C57BL/6NTac derived ES cell line. The target clone was screened by Long Range PCR and Southern blot. ES cell clones carrying the expected NF-κB1(p50）mutation were injected into E3.5 C57BL/6 blastocysts that were subsequently transferred into foster mothers. Knockin mutation was confirmed by sequencing tail DNA samples from offspring mice. Multiplex PCR genotyping used four primers to detect the

knockin alleles (primer 1, primer 2, primer 3, and primer 4). The following conditions of PCR reaction are used to detect Wild type and NF-κB1^{C59S} alleles: 94 °C, 5min; 41 cycles of 94 °C, 30 s; 58 °C, 30 s; 72 °C, 45 s; 72 °C, 5 min. Primers were obtained from Sangon Biotech, China, and the sequences are listed in Supplementary Figure 1. All of the offspring mice were maintained under a 12 light/12 dark cycle at a constant temperature of approximately 25°C and humidity between 35-75%. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the JinanUniversity. All surgery was performed under pentobarbital anesthesia, and all efforts were made to minimize mouse suffering.

Histology

Briefly, ovaries from 25-week-old wild-type (C57) or NF-κ B1^{C59S}mice were fixed in 4% paraformaldehyde at 4 °C for 24 hours. The specimens were then dehydrated, cleared in xylene, and embedded in paraffin wax. The embedded specimens were sectioned serially at 5 μm using a rotary microtome (Leica, Germany). The sections were either stained with hematoxylin and eosin (HE), periodic acid Schiff (PAS) reaction or Masson's trichrome dyes (Li et al., 2014). The PAS and Masson staining were used to reveal the presence of atretic follicle in the ovarian sections. The stained histological sections were photographed using an epifluorescence microscope and an attached camera (Olympus IX51, Leica DM 4000B) at 200× magnification.

Classification of developing follicles in ovarian sections

The follicles in the ovarian histological sections were developmentally staged according to their morphology as: primary, secondary, antral or atretic follicles. Briefly, Oocyte surrounded by a single or several layer/s of cuboidal granulosa cells were classified as a primary or secondary follicle, respectively. When an antrum was present, it was described as an antral follicle. The presence of zona pellucida remnants

was classified as an end-stage atretic follicle (Myers, Britt, Wreford, Ebling, & Kerr, 2004). Every 5th and 6th histological sections were selected for comparison and evaluation. Follicles were only counted if appeared in one histological section but not in the others (Myers, Britt, Wreford, Ebling, & Kerr, 2004).

Immunohistological Staining

Sections of mouse ovary were dewaxed, hydrated, incubated in citrate buffer (pH 6.0) and then heated in a microwave for antigen retrieval. Immunofluorescent staining was conducted on these treated sections using various antibodies. Briefly, the sections were incubated in the following primary antibodies diluted using PBT-NGS: IκBα (1:200, catalog#4814, Cell Signaling Technology, USA), p65 (1:200, catalog#6956, Cell Signaling Technology, USA), FSHR (1:100, catalog#22665-1-AP, Proteintech, China), LHCGR (1:100, catalog#BA3590, Boster, China), Ki67 (1:200, catalog BS1454, Bioworld, USA), Proliferating Cell Nuclear Antigen (PCNA) (1:200, catalog ab29, Abcam, USA), Fas (1:200, catalog#8023, Cell Signaling Technology, USA), FasL (1:100, catalog#PB0042, Boster, China), C-Caspase-3 (1:200, catalog#9664, Cell Signaling Technology, USA), α-SMA (1:400, catalog#ab5694, Abcam, USA) at 4 °C overnight. Following three 5 min washes in PBS, the sections were further incubated with goat anti-rabbit IgG or goat anti-mouse IgG conjugated Alexa Fluor 555 or 488 (1:1000, Life Technologies, USA) for 1 hour. The sections were counterstained with DAPI (1:1000, Life Technologies, USA) at room temperature for 30 min before examination. Photographs were taken of the stained histological sections using an epifluorescence microscope (Olympus IX51, Leica DM 4000B) at 200× magnification.

RNA isolation and quantitative PCR

Total RNA was isolated from 25-week-old mouse ovary or COV434 cells using a Trizol kit (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized to a final volume of 20 μl using iScriptTM cDNA Synthesis Kit (BIO-RAD, USA). Following reverse transcription, PCR amplification of the

cDNA was performed as described previously (Dugaiczyk et al., 1983; Maroto et al., 1997). SYBR® Green qPCR assays were then performed using a PrimeScriptTM RT reagent kit (Takara, Japan). All specific primers used are described in Supplementary Fig. 2. PCR reactions were performed in a Bio-Rad S1000TM Thermal cycler (Bio-Rad, USA) and ABI 7000 thermal cyclers, respectively. The housekeeping gene GAPDH was run in parallel to confirm that equal amounts of RNA used in each reaction. The expression of the genes was normalized to GAPDH, and the expression level was compared by ΔΔCt. The q-PCR result was representative of three independent experiments.

Western blot

25-week-old mouse ovary or COV434 cells was collected and lysed with CytoBuster™ Protein Extraction Reagent (#71009, Novagen). The total protein concentration was determined usinga BCA quantification kit (BCA01, DingGuoBioTECH, China). Samples containing equal amounts of protein were resolved by SDS-PAGE and then transferred to PVDF membranes (Bio-Rad, USA). The membranes were blocked with 5% Difco™ skimmed milk (BD) and then incubated with primary and secondary antibodies. The antibodies used were IκBα (1:1000, catalog#4814, Cell Signaling Technology, USA); p65 (1:1000, catalog#6956, Cell Signaling Technology, USA); FSHR (1:500, catalog#22665-1-AP, Proteintech, China); PCNA (1:1000, catalog ab29, Abcam, USA); Fas (1:1000, catalog#8023, Cell Signaling Technology, USA); C-Caspase3 (1:1000, catalog#9664, Cell Signaling Technology, USA); Phospho-AKT1 (Thr308) (1:1000, catalog#SB240133, Thermo Fisher scientific, USA); β-actin (1:2000, Proteintech, China); HRP-conjugated anti-mouse IgG and anti-rabbit IgG (1:3000, Cell Signaling Technology, USA). All primary and secondary antibodies used were diluted to 1:1000 and 1:2000 in 5% skimmed milk or BSA, respectively. The protein bands of interest were visualized using an ECL kit (#34079, Thermo Fisher Scientific Inc, USA) and GeneGnome5 (Syngene, UK). The staining intensity of the bands was determined and analyzed using Quantity One software (Bio-Rad, USA).

238

239 ***Cell lines and culture***

240 COV434 (human ovarian granulosa cells) was obtained from GuangZhouJennio
241 Biotech Co., Ltd, China. The cells were cultured in RPMI Medium 1640 basic (1X)
242 (Gibco) supplemented with 10% fetal bovine serum (Gaithersburg, MD, USA) in a
243 humidified incubator with 5% CO2 at 37 °C.

244

245 ***CCK8 Assays and Hoechst/PI staining***

246 COV434 cells, divided into Control, LPS (1μg/mL, 5μg/mL, 10μg/mL,Sigma,
247 USA), Bay 11-7082 (5μM, 10μM,calbiochem, Germany), were seeded into 96-well
248 plates. These cells (1×10^5 cells/mL) were maintained in RPMI Medium 1640 basic
249 (1×) + 10% fetal bovine serum at 37 °C and 5% CO_2. The cell viability was assessed
250 using CCK8 assay (cholecystokinin-8). Briefly, 10 μl of CCK8 reagent (Dojindo,
251 Kumamoto, Japan) was added to the 96-well plates and incubated for 12h, 24h and
252 48h at 37 °C. The absorbance values were measured at 450 nm using a Bio-Rad
253 model 450 microplate reader (Bio-Rad, USA). The cell viability was indirectly
254 determined by examining the ratio of the absorbance value of LPS-treated cells, and
255 Bay 11-7082-treated cells relative to the control cells.For Hoechst (1:1000, Sigma,
256 USA) / Propidium Iodide (PI, 1:1000, Sigma, USA) staining, the cells were cultured
257 and washed twice with cold PBS, and then incubated with Hoechst/PI for 45 min at
258 37°C in the dark.

259

260 ***Image acquisition and analysis***

261 Whole ovaries were photographed using a fluorescence stereomicroscope
262 (Olympus MVX10) and analyzed imaging software (Image-Pro Plus 6.0). The stained
263 sections of ovaries were photographed using an epi-fluorescent microscope (Olympus
264 IX51, Leica DM 4000B) at 200x and 400x magnification and analyzed with Olympus
265 software (Leica CW4000 FISH).

266 For quantification of proliferation, apoptosis and differentiation, the number of
267 IκBα$^+$, p65$^+$, FSHR$^+$, LHCGR$^+$, Ki67$^+$, PCNA$^+$, FasL$^+$, Fas$^+$, cleaved-Caspase-3$^+$

granulosa cells versus total $DAPI^+$ granulosa cells were counted for each follicle or visual field. The results were then compared between each group with the follicles only at the same developmental stage. For immunofluorescent staining of 25-week-old ovaries, total positive granulosa cells in secondary follicles or antral follicles were counted (Chen et al., 2015). Six ovaries of each experimental group were used.

Data Analysis

Data analyses and construction of statistical charts were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, USA). The results were presented as the mean value ($\bar{x} \pm$ SEM). Statistical analysis was performed using IBM SPSS Statistics 19.0 software. Statistical significance was determined using an independent samples t test, and non-parametric independent samples Kruskal-Wallis test. $P < 0.05$ was considered to be statistically significant.

Results

The dynamic change of NF-κB signaling pathway is associated with the transition from mouse ovarian secondary to antral follicles.

Immunofluorescent staining against IκBα (red, Fig. 1A-D1), p65 (green, Fig. 1E-H1) and p50 (brown, Fig. 1I-L) was implemented on transverse sections of 25-week-old C57 mouse ovaries to determine the expression pattern of NF-κB signaling pathway in developing ovarian follicles. The results demonstrated that IκBα , p65 and p50 are expressed in granulosa cells (Fig. 1). IκBα expression was increased from primordial follicles to secondary follicles, but reduced at antral follicles (Fig. 1A-D1), while p65 and p50 expression consistently increased until the follicles developed to antral follicles (Fig. 1E-L), which is schematically illustrated by the sketch in Fig. 1M. This indicates that NF-κB signaling is activated during the transition from secondary to antral follicles since IκBα is degraded, while p65 and p50 up-regulated (Razani, Reichardt, & Cheng, 2011).

To address the role of NF-κB signaling on the follicular transition, we generated active NF-κB mice through mutating serine into cysteine at the nos. 59 of sixth exon (Mitomo et al., 1994; Toledano, Ghosh, Trinh, & Leonard, 1993) (Fig. 2A). p50 immunofluorescent staining and western blot data showed that higher expression of p50 in active NF-κB ovaries than the one in wild-type (Supplementary Fig. 3; WT = 44.23 ± 3.110%, N=4; NF-κB1^{C59S} =74.85 ± 2.833%, N=4;Supplementary Fig. 3E; WT = 0.1730 ± 0.01159, N=3; NF-κB1^{C59S} =0.2857 ± 0.03122, N=3;Supplementary Fig. 3G). There was no obvious difference in appearance, ovary weight and surface area between the wild-type and active NF-κB mouse ovaries, although it appears that there were more blood vessels on the ovary surface of active NF-κB mice than the one on the wild-type (Fig. 2B;WT = 5.450±0.3114mg, N=10; NF-κB1^{C59S} =5.722 ± 0.2373mg, N=9;Fig. 2B1 ; WT = 3.660 ± 0.1288mm^2, N=5; NF-κB1^{C59S}= 3.904 ±0.09469mm^2, N=5; Fig. 2B2). To assess the angiogenesis in ovary, we implemented immunofluorescent staining against α-SMA (α-smooth muscle actin), the marker for vascular smooth muscle(Badid et al., 2002), on the ovary section, but we did not find the change of α-SMA expression between wild-type and active NF-κB ovaries (Supplementary Fig. 4; WT = 103.7 ± 5.333, N=6; NF-κB1^{C59S} = 116.7 ± 5.649, N=6; Supplementary Fig. 4G). Quantitative PCR data demonstrated the lower expression of IκBα and higher expression of p65 in active NF-κB ovaries than the one in wild-type (WT = 1.000 ± 0.02608, N=3; NF-κB1^{C59S} =0.5419 ± 0.05249, N=3; Fig. 2C; WT = 1.000 ± 0.1094, N=3; NF-κB1^{C59S} = 4.385 ± 0.4715, N=3; Fig. 2D). IκBα immunofluorescent staining showed that IκBα expression on granulosa cells (indicated by arrows) of the secondary and antral folliculeson ovary sections was also reduced in active NF-κB ovaries relative to wild-type (Fig. 2E), and the ratios of IκBα positive granulosa cell numbers in secondary and antral follicles of active NF-κB mice were significantly lower than the ones in wild-type mice (secondary: WT = 28.67 ± 2.404%, N=6; NF-κB1^{C59S} =10.42 ± 1.052%, N=6; Fig. 2E1; antral: WT = 6.450 ± 0.7325%, N=6; NF-κB1^{C59S} = 2.967 ± 0.4271%, N=6; Fig. 2E2). Furthermore, p65 immunofluorescent staining on ovary section demonstrated more p65 expression in cell nuclei of active NF-κB ovary follicles (indicated by arrows) than in wild-type

ovary follicles (Fig. 2F), and the ratio of p65-labelled NF-κB nuclear translocation in active NF-κB ovary follicles dramatically increased (WT = 42.38 ± 1.493%, N=6; NF-κB1^{C59S} = 65.92 ± 1.945%, N=6; Fig. 2F1). Meanwhile, western blot data manifested the similar results with the one from immunofluorescent staining. IκBα expression at the protein level decreased significantly in active NF-κB ovaries relative to wild-type (Fig. 2G; WT = 1.448 ± 0.1560, N=3; NF-κB1^{C59S} =0.9547 ± 0.04836, N=3; Fig. 2G1). All of those data imply that the mouse model of NF-κB signaling activation in the ovary follicles is well established.

Activation of NF-κB signaling raised the numbers of mouse ovarian antral follicles.

To assess the effect of elevated NF-κB signaling on follicular development, the numbers of differently developing ovarian follicles on the HE stained vertical sections of ovaries were counted (Fig. 3A), and the results showed that there was little change in the numbers of primary follicles, secondary follicles and corpus luteum except for the significant increase of antral follicle numbers between wild-type and active NF-κB mice (primary: WT = 2.500 ±0.4773, N=10; NF-κB1^{C59S} = 1.867 ± 0.3763, N=15; secondary: WT = 3.071 ± 0.6501, N=14; NF-κB1^{C59S} = 2.938 ± 0.3223, N=16; antral: WT = 7.000 ± 0.2572, N=17; NF-κB1^{C59S} = 9.923 ± 0.4995, N=13; corpus luteum: WT = 2.357 ± 0.1693, N=14; NF-κB1^{C59S} = 2.200 ± 0.4047, N=15; Fig. 3B). The folliculogenesis from primary follicles has been clearly associated with the regulation of endocrine signals, especially the estrogen converted from androgen, in which cytochrome P450 family (CYP) plays an important role (Fan et al., 2008). Here, quantitative PCR data showed that CYP11a1 and CYP19a1 expressions were up-regulated, while CYP17a1 expression remained unchanged in active NF-κB mice in comparison to wild-type mice (WT =1.000 ± 0.06945, N=3; NF-κB1^{C59S} = 1.586 ±0.1879, N=3; Fig. 3C; WT =1.000 ± 0.04619, N=3; NF-κB1^{C59S} = 0.9611 ±0.02268, N=3; Fig. 3D; WT =1.000 ± 0.08749, N=3;NF-κB1^{C59S} = 3.309 ± 0.2245, N=3; Fig. 3E). These data suggest that activation of NF-κB signaling promote the generation of antral follicles, and the subsequent high expression of CYP11a1 and CYP19a1 may contribute to the formation of estrogen.

Due to the obvious importance of gonadotropin on stimulating the ovarian development, the expressions of FSHR (Follicle Stimulating Hormone Receptor) and LHCGR (Luteinizing Hormone/Choriogonadotropin Receptor) were determined in the antral follicles of wild-type and active NF-κB mice using immunofluorescent staining. Immunofluorescent staining showed that both FSHR and LHCGR expressions in granulosa cells of active NF-κB mouse antral follicles were higher than the one in wild-type mice (Fig. 4A-B). This phenotype was confirmed by the quantitative PCR data (FSHR: WT = 1.000 ± 0.1424, N=3; NF-κB1^{C59S} = 2.659 ± 0.4128, N=3; LHCGR: WT = 1.000 ± 0.1017, N=3;NF-κB1^{C59S} = 3.331 ± 0.2323, N=3; Fig. 4C). Moreover, western blot data manifested that FSHR expression in active NF-κB mouse ovaries significantly up-regulated in comparison to wild-type at protein level (Fig. 4D; WT = 0.3120 ± 0.07679, N=3; NF-κB1^{C59S} = 0.6357 ± 0.02774, N=3; Fig. 4D1). This indicates that the activation of NF-κB signaling could promote the expressions of gonadotropin receptors in ovarian granulosa cells.

Activation of NF-κB signaling enhanced granulosa cell proliferation of mouse ovarian secondary and antral follicles.

Immunofluorescent staining against Ki67 and PCNA was implemented on the mouse transverse sections to assess the effects of NF-κB signaling on cell proliferation of the ovarian secondary and antral follicles (Fig. 5A). The results showed significantly higher expressions of both Ki67 (Secondary: WT = 28.60 ± 1.770%, N=6; NF-κB1^{C59S} = 40.83± 1.883%, N=6; Antral: WT = 30.08 ± 2.538%, N=6; NF-κB1^{C59S} = 46.50 ± 3.610%, N=6; Fig. 5A1) and PCNA (Secondary: WT = 48.22± 2.411%, N=6; NF-κB1^{C59S} = 51.08 ± 2.951%, N=6; Antral: WT = 41.33± 2.472%, N=6; NF-κB1^{C59S} = 56.75 ± 3.25%, N=6; Fig. 5A2) in the granulosa cells of NF-κB signaling-activated mouse secondary and antral follicles compared to wide-type. Furthermore, western blot data also demonstrated that there was a significantly increase of PCNA expression in NF-κB signaling-activated mouse ovaries compared to wild-type (Fig. 5B; WT = 0.4703 ± 0.02206, N=3; NF-κB1^{C59S} = 0.6063 ±0.02571, N=3; Fig. 5B1). Meanwhile, the expressions of bone morphogenetic

protein 15 (Bmp15) and growth differentiation factor 9 (Gdf9), both oocyte-secreted factors that are involved in regulation of the granulosa cell proliferation during follicular development (Reader et al., 2011), were determined in NF-κB signaling-activated and wild-type mouse ovaries using quantitative PCR. The results showed that activation of NF-κB signaling caused the enhanced expressions of both Bmp15 and Gdf9 in comparison to wild-type mice (Bmp15: WT = 1.000 ± 0.09598, N=3; NF-κB1^{C59S} = 2.024 ±0.2289, N=3; Gdf9: WT = 1.000 ± 0.1106, N=3; NF-κB1^{C59S} = 2.285 ± 0.1832, N=3; Fig. 5C). All of those data indicated that the accelerated process of conversion from secondary to antral follicles might partially due to promoted granulosa cell proliferation under the activation of NF-κB signaling.

Activation of NF-κB signaling regressed granulosa cell apoptosis of mouse ovarian secondary and antral follicles.

PAS and Masson staining were employed to identify the extent of NF-κB signaling-activated mouse ovarian antral follicles conversion into atretic follicles (indicated by asterisk) by their morphologic characteristics (Fig. 6A). The results showed that PAS and Masson staining-labelled atretic follicle numbers in NF-κB signaling-activated mouse ovaries were much less than the one in wild-type mice (WT = 14.69 ± 1.558, N=13; NF-κB1^{C59S} = 8.917 ± 0.9883, N=12; Fig. 6B). Follicular atresia is closely correlated with granulosa cell apoptosis (Lin & Rui, 2010). Therefore, immunofluorescent staining against FasL, Fas and C-capses3 was performed on the mouse transverse sections to evaluate the effects of NF-κB signaling on cell apoptosis of the ovarian secondary and antral follicles (Fig. 6C). The results demonstrated that FasL, Fas and C-capses3 were mainly expressed in granulosa cells (Fig. 6C), and activation of NF-κB signaling reduced the positive ratios of FsaL (Secondary: WT = 24.00 ±1.862%, N=6; NF-κB1^{C59S} = 16.17 ± 1.216%, N=6; Antral: WT = 25.35 ± 2.040%, N=6; NF-κB1^{C59S} = 11.42 ± 1.578%, N=6; Fig. 6D) and C-caspases3 (Secondary: WT = 38.17 ±3.664%, N=6; NF-κB1^{C59S} = 27.00 ± 1.862%, N=6; Antral: WT = 41.67 ± 2.813%, N=6; NF-κB1^{C59S} = 25.20 ± 3.175%, N=6; Fig. 6F) expression on the granulosa cells of secondary and antral follicles. Meanwhile,

Fas expressionon the granulosa cells of antral follicles was also lower in comparison to wild-type mice (WT = 16.95 ± 0.8597%, N=6; NF-κB1^{C59S} = 9.85 ± 1.013%, N=6; Fig. 6E). Using quantitative PCR, we determined the mRNA expressions of a number of cell apoptosis-related factors. Expression of Faswas unchanged, Bcl-2 increased and expressions of FasL, Bax, PUMA, and P53 were reduced in NF-κB signaling-activated mouse ovaries compared to wild-type ones (Fas:WT = 1.000 ± 0.1186, N=3; NF-κB1^{C59S} = 1.003 ± 0.09953, N=3; Fasl: WT = 1.000 ± 0.04608, N=3; NF-κB1^{C59S} = 0.4009 ± 0.04047, N=3; Bcl-2: WT = 1.000 ± 0.03217, N=3; NF-κB1^{C59S}= 8.703 ± 0.5728, N=3; Bax:WT = 1.000 ± 0.04390, N=3; NF-κB1^{C59S} = 0.5819 ± 0.08119, N=3; PUMA: WT = 1.000 ± 0.09993, N=3; NF-κB1^{C59S} = 0.4077 ± 0.03884, N=3; P53: WT = 1.000 ± 0.07817, N=3; NF-κB1^{C59S} = 0.5967 ± 0.09822, N=3; Fig. 6G). Similarly, western blot data showed the expressions of Fas and C-capases3 at protein level were also reduced in NF-κB signaling-activated mouse ovaries compared to wild-type (Fig. 6H; Fas: WT = 2.102 ± 0.1842, N=3; NF-κB1^{C59S} = 0.8573 ± 0.04725, N=3; c-caspase3: WT = 0.3753 ± 0.04245, N=3; NF-κB1^{C59S} =0.1270 ± 0.02857, N=3; Fig. 6H1). All the data suggest that elevated NF-κB signaling suppress granulosa cell apoptosis, which in turn hinder the process of follicular atresia.

LPS-induced NF-κB signaling activation promoted cell proliferation and differentiation-related gene expressions in granulosa cells in vitro.

To further investigate the role of NF-κB signaling in granulosa cells, we enhanced NF-κB signaling in COV434 cells, a human ovarian granulosa tumor cell line, and exposed them to lipopolysaccharides (LPS). Cell Counting Kit-8 (CCK-8), a colorimetric assay kit, was employed to assess COV434 cell proliferation and cytotoxicity to various concentrations of LPS (Fig. 7A). The results showed that the exposure to 1μg and 5μg/ml LPS for 48 hours could increase cell viability, but 10μg/ml LPS for 48 hours suppressed cell viability (24h: Control =100.0 ± 1.657%, N=6; LPS 1μg/ml = 101.3 ± 6.392%, N=6;LPS 5μg/ml = 93.45 ± 2.489%, N=6; LPS 10μg/ml = 103.4 ±1.740%, N=6; 48h: Control = 100.0 ± 5.217%, N=6; LPS 1μg/ml =

138.2 ± 9.573%, N=6; LPS 5μg/ml = 142.4 ±14.27%, N=6; LPS 10μg/ml = 108.5 ± 3.757%, N=6; Fig. 7A). P65 immunofluorescent staining showed that 1μg/ml LPS exposure increased p65 nuclear translocation in COV434 cells (Fig. 7B; Control = 11.18 ± 2.475%, N=5; LPS = 27.12 ± 2.057%, N=5; Fig. 7B1).Western blot data showed that 1μg/ml LPS exposure increased p65 expression and reduced IκBα expression (Fig. 7C; p65: Control = 0.2827 ± 0.03636, N=3; LPS = 0.4960 ± 0.03523, N=3; IκBα: Control = 1.098 ± 0.07959, N=3; LPS = 0.8256 ± 0.02862, N=3; Fig. 7C1). Those data indicates that NF-κB signaling was indeed activated by the exposure of 1μg/ml LPS in COV434 cells *in vitro*. To assess the correlation between activation of NF-κB signaling and FSH-regulated granulosa cell activities, we determined the expressions of FSH receptor, phosphate-AKT and PCNA using western blotting in presence of 1μg/ml LPS. The results showed that 1μg/ml LPS exposure increased the expressions of FSH receptor, phosphate-AKT and PCNA at the protein level in COV434 cells (Fig. 7D; FSHR: Control = 0.4303 ±0.02537, N=3; LPS = 0.5080 ± 0.01172, N=3; P-AKT: Control = 0.2313 ±0.01041, N=3; LPS = 0.2935 ± 0.01843, N=3; PCNA: Control = 0.4703 ± 0.02063, N=3; LPS = 0.5497 ± 0.01889, N=3; Fig. 7D1). Meanwhile, quantitative PCR was employed to determine the expressions of NF-κB signaling-related genes in COV434 cells exposed to LPS. The results showed that 1μg/ml LPS exposure regressed IκBα expression; did not change TRAF6 expression; increased TNFα, IL-6, IL-8 expressions (IκBα: Control = 1.000 ± 0.09502, N=3; LPS = 0.4203 ± 0.08182, N=3; TRAF6: Control = 1.000 ± 0.04847, N=3; LPS =0.8216 ±0.1663, N=3; TNFα: Control = 1.000 ± 0.1976, N=3; LPS = 3.283 ± 0.4710, N=3; IL-6: Control = 1.000 ± 0.09318, N=3; LPS = 3.500 ± 0.6464, N=3; IL-8: Control = 1.000 ± 0.04035, N=3; LPS = 5.028 ± 0.6371, N=3; Fig. 7E).

To further verify these observations, FSHR expression and COV434 cell proliferation was determined when NF-κB signaling was blocked through application of BAY 11-7082, a NF-κB inhibitor (Fig. 8). BAY 11-7082 is a specific inhibitor inducing IκBα phosphorylation, which can suppress the NF-κB signaling pathway(Kamthong & Wu, 2001).The BAY 11-7082 concentration used in the range of 2.5-10 μM did not display the signs of cytotoxicity in previous study (Xia et al.,

2018). So 5μM BAY 11-7082 treatment for 12 hours was chosen since cell proliferation and cytotoxicity assays with CCK-8 indicated no effect on COV434 cell viability (12h: Control = 100.0 ± 3.650%, N=6; BAY 11-7082 5μM = 91.52 ± 5.050%, N=6; BAY 11-7082 10μM = 81.73 ±6.097%, N=6; 24h: Control = 100.0 ± 1.891%, N=6; BAY 11-7082 5μM = 73.77±2.696%, N=6; BAY 11-7082 10μM = 64.49 ± 4.205%, N=6; 48h: Control = 100.0 ±3.160%, N=6; BAY 11-7082 5μM = 77.49 ± 1.576%, N=6; BAY 11-7082 10μM = 51.85 ± 2.810%, N=6; Fig. 8A). p65 and FSHR immunofluorescent staining confirmed 1μg/ml LPS exposure promoted p65 transfer into the nucleus and promoted FSHR expression (Fig. 8B-B1). However, the reduction of p65 expression indicated the successful blockage of NF-κB signaling with BAY 11-7082and the subsequent NF-κB signaling blockage lead to the regression of FSHR expression in COV434 cells (Fig. 8B; Control =41.93 ±4.294%, N=4; LPS = 55.28 ± 3.161%, N=4; BAY 11-7082 = 29.88 ± 2.207%, N=4; Fig. 8B1). Meanwhile, 1μg/ml LPS exposure promoted COV434 cell proliferation and suppressed COV434 cell apoptosis, but blockage of NF-κB signaling with BAY 11-7082 suppressed COV434 cell proliferation (Fig. 8C; Control = 43.05 ± 2.235%, N=4; LPS = 52.23 ± 2.925%, N=4; BAY 11-7082 = 27.03± 2.235%, N=4; Fig. 8C1) and promoted COV434 cell apoptosis (Fig. 8D; Control = 5.233 ± 0.2333%, N=3; LPS = 2.603 ± 0.5323%, N=3; BAY 11-7082 = 74.07 ± 1.848%, N=3; Fig. 8D1). All of the data suggest that LPS-activated NF-κB signaling could be directly involved in FSH-mediating granulosa cell viability and proliferation.

Discussion

NF-κB signaling is involved in numerous cellular events under normal physiological states and disorders. However, the role of of NF-κB signaling in follicular development is not well understood. To address this issue, the expression patterns of IκB, p65 and p50, the marker molecules of NF-κB signaling, were determined in wild-type mouse developing ovarian follicles (Fig. 1). Degradation of IκB proteins induced by any signaling could activate NF-κB(Solt & May, 2008). As

one of NF-κB transcription factor family's five components, p65 forms heterodimers and translocates from the cytoplasm to the nucleus in the NF-κB signaling pathway presented in most cell types (Basseres & Baldwin, 2006). IκB expression increased from primordial to secondary follicles, but it decreased when ovarian follicles developed into antral follicles, while p65 and p50 expression constantly went up at all stages through to antral follicles (Fig. 1A-L), implying that the activation of the NF-κB signaling pathway in granulosa cells probably promotes the conversion of secondary follicles into antral follicles (Fig. 1M). To investigate this assumption, active NF-κB signaling transgenic mice were generated by using knockin approach. Cysteine at the number 59 amino acid of the sixth exon (NF-κB1, p50 gene) was converted into serine (Fig. 2A), as previously reported (Toledano, Ghosh, Trinh, & Leonard, 1993). In the activated NF-κB signaling mouse ovaries, lower expression of IκB in granulosa cells (Fig. 2E), higher expression of p50 in granulosa cells (Supplementary Fig. 3) and an increase of p65 nuclear translocation in granulosa cells was observed (Fig. 2F). This proves NF-κB signaling was activated in granulosa cells of secondary follicles and antral follicles. This physiological activation by just increasing p50 DNA-binding activity was more meaningful than the other way, such as continuously activating NF-κB by exogenous signals.

Next, the mouse ovarian follicular development was determined following the activation of NF-κB signaling by carefully counting the numbers of the variety of developing follicles on HE stained transverse sections (Fig. 3A). This indicated that there were no significant alterations of the numbers of primary, secondary follicles and corpus luteum except for antral follicles between wild-type and active NF-κB mice (Fig. 3B). CYP19a1, FSH and FSHR are responsible for estradiol production in ovarian granulosa cells through the cAMP-PKA pathway (Kim, Pyun, Cha, Ko, & Kwack, 2011). Therefore, the observed increase of CYP11a1 and CYP19a1expressions in active NF-κB ovaries compared to wild-type (Fig. 3C-E) also suggested the promotion of antral follicular development by activation of NF-κB signaling. FSH signals activate the heptahelical G protein-coupled FSHR in granulosa cells to promote follicular maturation(Park et al., 2005). Folliculogenesis involves the

activation of a small number of primordial follicles which then develop and pass through the primary, secondary and antral stages. These follicle stages involves very precise cellular and molecular interactions (Roche, 1996; Scaramuzzi et al.). Granulosa cells are normally indispensable for inducing and supporting the development of the ovarian follicles (Li et al., 1998; Porter, Vickers, Cowan, Huber, & Quirk, 2000). It is generally recognized that the interaction of autocrine and paracrine effectors, as well as FSH and LH ultimately determines the developmental fate of the developing follicles (Roche, 1996; Scaramuzzi et al., 2011). In this study, higher FSHR and LHCGR expressions were found in active NF-κB ovaries compared to wild-type (Fig. 4). This quantitative alteration of antral follicular numbers and the enhanced corresponding steroid hormone receptor confirm that NF-κB signaling is somehow involved in regulating the transition from ovarian secondary to antral follicles.

Using Ki67 and PCNA immunofluorescent staining, it was demonstrated that the activation of NF-κB signaling definitely enhanced the granulosa cell proliferation in secondary and antral follicles (Fig. 5A-A2). Western blotting result also showed that higher PCNA expression in active NF-κB ovaries compared to wild-type (Fig. 5B-B1). These data indicated that the granulosa cells' ability to respond to FSH-stimulated cell proliferation was dramatically promoted under the activation of NF-κB signaling. There is no doubt that the vigorous granulosa cell proliferation in turn also accelerates the maturation of ovarian follicles (Maruo et al., 1999). In addition, folliculogenesis at later stages of development is largely mediated by oocyte-granulosa-theca cell interactions. Granulosa cells and cumulus cells, the two anatomically and functionally obvious layers, are also responsible for nurturing oocyte development and subsequent acquisition. That is to say, oocyte and granulosa cell/cumulus cell communication is bidirectional, which is illustrated by potent growth factors secreted by oocytes directly influencing the differentiation and function of granulosa cells. Gdf9 and Bmp15 are the most important two oocyte-secreted factors (OSFs) enabling oocytes apparent ability to regulate their neighboring somatic cells and guiding them to implement functions (Gilchrist, Lane, & Thompson, 2008). Furthermore, during follicular

transitional stage, granulosa factors promote the recruitment of theca cells from stromal cells, while oocyte-derived Gdf9 maintains follicular development from pre-antral to antral stage by regressing granulosa cell apoptosis (Orisaka, Tajima, Tsang, & Kotsuji, 2009; Thomas & Vanderhyden, 2006). In this study, Bmp15 and Gdf9 were up-regulated in active NF-κB signaling mice (Fig. 5C), implying activation of NF-κB signaling enhances the FSH-induced response of granulosa cells on differentiation and proliferation. Meanwhile, PAS and Masson staining clearly showed that activation NF-κB signaling suppressed the process of follicular atresia (Fig. 6A-B), which was further confirmed by the down-regulation of Fas/Fasl and C-capsese3 in granulosa cells of secondary and antral follicles in active NF-κB mice (Fig. 6C-H). This finding also verifies the role of NF-κB signaling on follicular maturation.

NF-κB signaling is the initial cellular responder to harmful stimuli, which could include bacterial lipopolysaccharides (LPS), reactive oxygen species (ROS), tumor necrosis factor alpha (TNFα), interleukin 1-beta (IL-1β) and ionizing radiation (Chandel, Trzyna, McClintock, & Schumacker, 2000). This allows us to validate the observation mentioned above in active NF-κB mice using COV434, a granulosa cell line, exposed to LPS in vitro (Fig. 7). In this study, 1μg/mland 5μg/ml LPS exposure stimulated COV434 cell viability, but 10μg/ml LPS exposure suppressed it. This indicatesthat only certain levels of NF-κB signaling changes promote granulosa cell viability. Moreover, 1μg/ml LPS exposure promoted p65 nuclear translocation, IκBa down-regulation, FSH receptor, TNFα, IL-6 and IL-8 up-regulation (Fig. 7). In order to confirm the level of NF-κB signaling that could stimulate granulosa cell viability, NF-κB signaling was blocked by the addition of a NF-κB inhibitor, BAY11-7082, which was added into the culture medium of COV434 cells. The BAY11-7082 suppressed p65 nuclear translocation and inhibited cell survival, but this may also be through the regulation of FSHR expression (Fig. 8). All the data suggest that NF-κB signaling is closely associated with granulosa cell proliferation, apoptosis and differentiation, which are regulated by FSHR and its downstream products at the late stage of follicular development.

In this study, the role of NF-κB signaling on ovarian follicular development is for the first time revealed as illustrated in Fig. 9. Briefly, NF-κB signaling in granulosa cells of developing follicles is particularlyactivated during the transition from secondary to antral follicles. Activated NF-κB signaling suppresses apoptosis and promotes proliferation and differentiation of granulosa cells, which also mostly occurs during the transition from secondary to antral follicles. This study provides, to our knowledge, the first experimental evidence that NF-κB signaling is involved in the control of follicular development through FSHR and its corresponding target molecules. Nevertheless, more experiments are needed to be precisely conducted before the full role of the physiological functions of NF-κB signaling in ovarian follicular development can be completely addressed.

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Competing Financial Interest

The authors have declared that no competing interests exist.

Author contributions

J.X. performed the experiments and collected the data; G.W. and X.Y. designed the study and analyzed the data; X.L. performed the experiments; Y.B. and L.W. critically read the manuscript. X.Y. wrote manuscript.

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Figure legends

Fig. 1. IκBα, p65 and p50 expression patterns in developing mouse ovarian follicles

A-D: Representative IκBαimmunofluorescent micrographs of primordial follicle (A), primary follicle (B), secondary follicle (C) and antral follicle (D) on the ovarian transverse sections of 25-week C57 mice. **A1-D1**: Merged images of DAPI staining and A-D respectively. **E-H**: Representative p65immunofluorescent micrographs of primordial follicle (E), primary follicle (F), secondary follicle (G) and antral follicle (H) on the ovarian transverse sections of 25-week C57 mice. **E1-H1**: Merged images of DAPI staining and E-H respectively. **I-L**: Representative p50immunohistochemistry micrographs of primordial follicle (I), primary follicle (J), secondary follicle (K) and antral follicle (L) on the ovarian transverse sections of 25-week C57 mice. **I1-L1**: Negative of immunohistochemistry. **M**: Sketches illustrating the expression patterns of IκBα, p65 and p50 in mouse developing ovarian follicles. Abbreviation: Pdf, primordial follicle; PF, primary follicle; SF, secondary follicle; AF, antral follicle; TC, theca cells; GC, granulosa cells; ZP, zona pellucida. Scale bars = 30μm in A-L1.

Fig. 2. Establishing active NF-κB transgenic mice

A: Transgene schematic through altering NF-Kb1(p50)sequence of bases on 6th exon.**B**: Representative ovary appearance of 25-week-old wild-type and active NF-κB transgenic mice. **B1-B2**: The scatterplot and bar chart comparing the ovarian weights (B1) and surface area (B2) between wild-type and active NF-κB group. **C-D**: Bar charts showing the quantitative PCR data about the mRNA expressions (normalized to GAPDH) of IκBα (C) and p65 (D) between wild-type and active NF-κB mouse ovaries. **E**: Representative IκBαimmunofluorescent micrographs of secondary and antral follicle (left panels: IκBα staining only; middle panels: IκBα + DAPI staining; right panels: high magnification of the dotted areas in the middle panels) on the ovarian transverse sections of 25-week wild-type and active NF-κB mice.**E1-E2**: Bar

charts showing the ratio comparisons of IκBα positive cell numbers in total DAPI positive cells of secondary follicles (E1) or antral follicles (E2) between wild-type and active NF-κB mouse ovaries. **F**: Representative p65immunofluorescent micrographs of secondary (upper panel: wild-type; lower panel: active NF-κB) on the ovarian transverse sections of 25-week wild-type and active NF-κB mice.**F1**: The bar chart showing the percentages of p65 expressing in ovarian granulosa cell nucleuses between wild-type and active NF-κB mouse ovaries. **G-G1**: Western blot showing the IκBα expression at protein level in wild-type and active NF-κB mouse ovaries (G). Bar chart (G1) showing the relative comparison of IκBα expression(normalized to β-actin)revealed by western blot. Abbreviation: SF, secondary follicle; AF, antral follicle; GC, granulosa cells; ZP, zona pellucida. *p<0.05 , **p<0.01 and ***p<0.001 indicate significant difference between control and experimental groups. Scale bars = 400μm in B; 20μm in E-F.

Fig. 3. Alteration on the ovarian follicles in the active NF-κB mouse ovaries

A: Representative HE stained ovarian transverse sections from 25-week-old wild-type (left panel) and active NF-κB (right panel) mice. **B**: Bar chart showing the comparisons of various ovarian follicle numbers between wild-type and active NF-κB mouse ovaries. **C-E**: Quantitative PCR data showing the relative mRNA expressions (normalized to GAPDH) of CYP11a1 (C), CYP17a1 (D) and CYP19a1 (E) between wild-type and active NF-κB mouse ovaries. **F**: Sketches illustrating the potential target point of NF-κB pathway during the development of ovarian follicles. Abbreviation: SF, secondary follicle; AF, antral follicle. *p<0.05 and ***p<0.001 indicate significant difference between control and experimental groups. Scale bars = 200μm in A.

Fig. 4. FSHR and LHCGR expression in ovarian follicles

A-B: Representative micrographs of antral follicles immunofluorescently stained for FSHR (Follicle Stimulating Hormone Receptor) (A) and LHCGR (Luteinizing Hormone/Choriogonadotropin Receptor) (B) in the wild-type and active NF-κB

mouse ovaries. The low panels were the higher magnification of dotted areas in each immunofluorescence. **C**: Quantitative PCR data showing the relative mRNA expressions (normalized to GAPDH) of FSHR and LHCGR in wild-type and active NF-κB mouse ovaries. **D-D1**: Western blot showing the FSHR expression at protein level in wild-type and active NF-κB mouse ovaries (D). The bar chart (D1) showing the relative comparison of FSHR expression(normalized to β-actin)revealed by western blot. Abbreviation: GC, granulosa cells. *p<0.05 and ***p<0.001 indicate significant difference between control and experimental groups. Scale bars = 20μm in A-B.

Fig. 5. Granulosa cell proliferation using immunofluorescent or quantitative PCR and western blot

A: Representative micrographs of secondary and antral ovarian follicles immunofluorescently stained for Ki67 (the upper two panels) and PCNA (the lower two panels) to demonstrate the extent of granulosa cell proliferation in the wild-type and active NF-κB mouse ovarian follicles. The low panels were the higher magnification of dotted areas in each immunofluorescence. **A1-A2**: Bar charts comparing the percentages of $Ki67^+$ (A1) and $PCNA^+$ (A2) granulosa cells in secondary and antral follicles of 25-week-old wild-type and active NF-κB mouse ovarian follicles. **B-B1**: Western blot showing the PCNA expression at protein level in wild-type and active NF-κB mouse ovaries (B). The bar chart (B1) showing the relative comparison of PCNA expression (normalized to β-actin) revealed by western blot. **C**: Quantitative PCR data showing the relative mRNA expressions (normalized to GAPDH) of Bmp15 and Gdf9 in wild-type and active NF-κB mouse ovaries. Abbreviation: SF, secondary follicle; AF, antral follicle; GC, granulosa cells. *p<0.05 , **p<0.01 and ***p<0.001 indicate significant difference between control and experimental groups. Scale bars = 20μm in A.

Fig. 6. Granulosa cell apoptosis using immunofluorescent or quantitative PCR and western blot

A-B: Representative micrographs of 25-week-old wild-type (the left panel) and active NF-κB (the right panel) mouse ovarian transverse sections histochemically stained with PAS (the upper panel) or Masson (the lower panel) (A). Bar chart comparing the atretic follicle numbers between wild-type and active NF-κB mouse ovaries (B). **C**: Representative micrographs of secondary and antral follicles immunofluorescently stained for FasL (the first and second panels, in which the second panel is merge of FasL and DAPI staining), Fas (the third and forth panels, in which the forth panel is merge of Fas and DAPI staining), and C-caspase3 (the fifth panel - the merge of C-caspase3 and DAPI staining) in the wild-type and active NF-κB mouse ovarian follicles. **D-F**: Bar charts comparing the percentages of $FasL^+$ (D), Fas^+ (E), and C-caspase3^+ (F) granulosa cell ratios in every secondary and antral follicle between wild-type and active NF-κB mouse ovaries. **G**: Quantitative PCR data showing the relative mRNA expressions (normalized to GAPDH) of apoptosis-related genes including Fas, FasL, Bcl-2, Bax, PUMA and P53 in wild-type and active NF-κB mouse ovaries. **H-H1**: Western blot showing the Fas and C-caspase3 expression at protein level in wild-type and active NF-κB mouse ovaries (H). The bar chart (H1) showing the relative comparison of Fas and C-caspase3 expression (normalized to β-actin) revealed by western blot. Abbreviation: SF, secondary follicle; AF, antral follicle; Atf, atretic follicle; ZP, zona pellucida; GC, granulosa cells. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ indicate significant difference between control and experimental groups. Scale bars = 50μm in A and C.

Fig. 7. Cell viability and the expressions of NF-κB pathway crucial molecules in COV434 cells in presence of LPS

A: Cell Counting Kit-8 (CCK-8) was employed to determine cell viabilities of COV434 cells exposed to the various concentrations of LPS. **B-B1**: Representative micrographs of COV434 cells immunofluorescently stained p65 and counterstained DAPI in control (the upper panel) and LPS-treated (the lower panel) group. Bar chart comparing the p65 nuclear translocation ratios (percentage) in all cell between control and LPS-treated group (B1). **C-C1**: Western blot showing the p65 and IκBα

expression at protein level in COV434 cells from control and LPS-treated group (C). The bar chart (C1) showing the relative comparison of p65 and IκBα expression revealed by western blot. **D-D1**: Western blot showing the FSHR, P-AKT and PCNA expression at protein level in COV434 cells from control and LPS-treated group (D). The bar chart (D1) showing the relative comparison of FSHR, P-AKT and PCNA expression (normalized to β-actin) revealed by western blot. **E**: Quantitative PCR data showing the relative mRNA expressions (normalized to PPIA) of IκBα, TRAF6, TNFα, IL-6 and IL-8 in COV434 cells from control and LPS-treated group. *$p<0.05$ and **$p<0.01$ indicate significant difference between control and experimental groups. Scale bars = 20μm in B.

Fig 8. Cell viability, proliferation and apoptosis of COV434 cells following blockage of NF-κB pathway.

A: Cell Counting Kit-8 (CCK-8) was employed to determine cell viabilities of COV434 cells exposed to the various concentrations of Bay11-7082 (NF-κB inhibitor). **B-B1**: Representative micrographs of COV434 cells immunofluorescently stained p65 (green), FSHR (red) and counterstained DAPI (blue) in control (the first panel), LPS-treated (the secondary panel) and Bay11-7082-treated (the third panel) group (B). Bar chart (B1) comparing the percentage of $FSHR^+$ granulosa cells in total cells among control, LPS-treated and Bay11-7082-treated group. **C-C1**: Representative micrographs of COV434 cells immunofluorescently stained Ki67 (red) and counterstained DAPI (blue) in control (the first panel), LPS-treated (the secondary panel) and Bay11-7082-treated (the third panel) group (C). Bar chart (C1) comparing the percentage of $Ki67^+$ granulosa cells in total cells among control, LPS-treated and Bay11-7082-treated group. **D-D1**: Representative micrographs of COV434 cells (bright-field), immunofluorescently stained PI (red) and counterstained DAPI (blue) in control (the first panel), LPS-treated (the secondary panel) and Bay11-7082-treated (the third panel) group (D). Bar chart (D1) comparing the percentage of PI^+ granulosa cells in total cells among control, LPS-treated and Bay11-7082-treated group. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ indicate significant

909 difference between control and experimental groups. Scale bars = 20μm in B; 30μm in
910 C-D.
911
912 ***Fig 9. Involvement ofNF-κB pathway is in the regulation of mouse folliculogenesis.***
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