Follicle stimulating hormone inhibits the expression of p53 up-regulated modulator of apoptosis induced by reactive oxygen species through PI3K/AKT in mouse granulosa cells

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Short title: Follicle stimulating hormone inhibits p53 up-regulated modulator of apoptosis expression in mouse granulosa cells

Summary:

In mammalian ovaries, follicular atresia occurs periodically and destroys almost all the follicles in the ovary. Follicle-stimulating hormone (FSH) acts as the primary survival factor during follicular atresia by preventing apoptosis in granulosa cells (GCs). Many studies have demonstrated that oxidative stress-induced apoptosis is a main cause of follicular atresia. Reactive oxygen species (ROS)-induced GCs apoptosis is regulated by a variety of signaling pathways involving numerous genes and transcription factors. Therefore, we examined whether FSH inhibits the expression of p53 up-regulated modulator of apoptosis (PUMA) induced by reactive oxygen species (ROS) through phosphoinositide 3-kinase (PI3K) / protein kinase B (AKT) in mouse GCs. In vivo study: thirty-two-mice were randomly assigned to four groups and given FSH. We found that FSH can inhibit the 3-nitropropionic acid (3-NP) induced apoptosis and PUMA expression in mRNA level. Moreover, In vitro experiment, we found that FSH can inhibit the H2O2-induced apoptosis and PUMA expression in mRNA level. Additionally, we also found that PI3K/AKT inhibitor LY294002 abolished the downregulation of PUMA mRNA by FSH in vitro, In conclusion, FSH inhibit the expression of PUMA induced by ROS through PI3K/AKT pathway in vivo and vitro.

Keywords: Follicle stimulating hormone • Oxidative stress • PI3K/AKT • Granulosa cells.

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Introduction

More than 99% of the mammalian ovarian follicles undergo degeneration during growth and development, a phenomenon called as follicular atresia (Asselin *et al.,* 2000). Previous studies have demonstrated that follicular atresia is associated with granulosa cells (GCs) apoptosis, where DNA fragmentation, upregulation of pro-apoptotic gene expression and activation of caspases are observed (Jiang *et al.* 2003). Apoptosis can be induced by extrinsic as well as intrinsic factors in response to oxidative stress and damage (Valdez *et al.* 2005). Members of the B cell lymphoma 2 (Bcl-2) gene family have a central role in regulating programmed cell death by controlling pro-apoptotic and anti-apoptotic intracellular signals. Studies have suggested a close association between Bcl-2 family proteins and GCs apoptosis (Yang *et al.* 2017; Chowdhury *et al.* 2013). p53-upregulated modulator of apoptosis (PUMA) was originally identified as a BH3-only Bcl-2 subfamily protein. PUMA exerts its pro-apoptotic functions by inserting its BH-3 domain into the hydrophobic pocket created by folding the BH1, BH2, and BH3 domains of anti-apoptotic Bcl-2 family members. This insertion relieves the inhibitory effect of anti-apoptotic Bcl-2 family members on Bax and/or Bak7, and promotes apoptosis (Roychoudhury *et al*. 2016).

Reactive oxygen species (ROS) are short-lived and highly reactive molecules. The generation of ROS in cells exists in equilibrium with a variety of antioxidant defences. At low to modest doses, ROS are considered to be essential for regulation of normal physiological functions involved in development such as cell cycle progression and proliferation, differentiation, migration and cell death. ROS also play an important role in the immune system, maintenance of the redox balance and have been implicated in activation of various cellular signalling pathways (such as PI3K/AKT signalling pathways). Excess cellular levels of ROS cause damage to proteins, nucleic acids, lipids, membranes and organelles, which can lead to activation of cell death processes such as apoptosis. Apoptosis is a highly regulated process that is essential for the development and survival of multicellular organisms. Oxidative stress is a critical factor in follicular atresia (Shen M *et al*. 2000)). Inhibition of ROS-induced GCs apoptosis will prevent abnormal follicular atresia, which can be used as a therapeutic tool to alleviate reproductive failure to improve the economy of production efficiency. Therefore,

elucidating mechanisms of GCs apoptosis is vital for the development of new drugs that inhibit GCs apoptosis and cure abnormal atresia.

The development and maturation of follicles is a complex process which is regulated by gonadotropins and intraovarian regulators (Droge *et al.* 2008, Dierich *et al.* 1998). Particularly, follicle stimulating hormone (FSH) is required for the production of estrogen (Feng *et al.* 2017), growth and development of antral follicles (Bishop *et al.* 2017), and the selection of dominant follicles (DFs) (Fauser *et al.* 1997). These physiological responses to FSH are achieved by activating several signaling cascades in GCs, including protein kinase A (PKA), protein kinase B (PKB/AKT), p38 mitogen-activated protein kinase (p38-MAPK), and extracellular signal-regulated kinases 1 and 2 (ERK1/2), which modulate more than 100 target genes (Hunzicker *et al*. 2006). FSH was identified as one of the major survival factors for antral follicles for its ability to antagonize apoptosis in GCs (Chun *et al*. 1996,). However, its target genes and the exact mechanism for protection of granulose cells from apoptosis remains to be understood

Excessive generation of ROS by external stimuli can activate many apoptotic pathways, such as PI3K/AKT, c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase, and nuclear factor-κB signaling (Simon *et al*. 2000; Finkel *et al.* 2000; Ki *et al*. 2013, Deng *et al*. 2017). Endoplasmic reticulum stress induced by adenosine triphosphate deficiency could lead to the generation of ROS and upregulate mRNA and protein expression of PUMA in neuronal cells (Steckley *et al*. 2007). However, apoptotic signaling pathways are often tissue specific and respond to condition specific triggers. Few studies have systematically demonstrated the involvement and function of PUMA in the progression of oxidative stress-induced GCs apoptosis.

In our previous study, PUMA was shown to play a pivotal role in oxidative stress-induced GC apoptosis, which was regulated by forkhead box protein O1 (FOXO1) transcription factors. However, it is unknown whether PUMA is regulated by FSH during oxidative stress-induced GCs apoptosis. In our present study, we investigated whether PUMA is regulated by FSH through examining PUMA mRNA expression and protein level in vivo as well as in vitro. Additionally, we investigated the role of PI3K/AKT pathway on the inhibition of PUMA by FSH.

Methods

Ethical approval

All animal procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University. The protocols were reviewed and approved, and the project number 2011CB100802 was assigned. The slaughter and sampling procedures strictly followed the 'Guidelines on Ethical Treatment of Experimental Animals' (2006) no. 398 established by the Ministry of Science and Technology, China and the 'Regulation regarding the Management and Treatment of Experimental Animals' (2008) no. 45 set by the Jiangsu Provincial People's Government.

Animal and experimental procedures

In vivo study. Three to 4-week-old female (Imprinting control region) ICR mice (Nanjing Qinglongshan Experimental Animal Center) were housed (n=8 per group):, five per cage, in a temperature controlled (22±2 °C) room with a 12: 12 h light: dark cycle (lights on from 07 00 to 1900 hours) and free access to water and food. The control group, which received an equal volume of phosphate-buffered saline (PBS, pH 7.4; Sigma, St Louis, Missouri); the 3NP group, which was treated with 12 mg/kg 3 nitropropionic acid (3-NP) diluted with PBS twice daily for 1 week. the FSH group, which was treated with murine-FSH (Ningbo Second Hormone Factory, Ningbo, China) twice daily for 2 days at a dose of 10 IU on day 1 and 5 IU on day 2; the 3NP/FSH group, which was treated with twice daily for 2 days at a dose of 10 IU on day 1 and 5 IU on day 2 and 12 mg/kg 3-NP diluted with PBS twice daily for 1 week. Mouse GCs were isolated from dominant follicles (DFs; >200 μm) in the left ovaries of each mouse, for qRT-PCR. The right ovaries were fixed with 4% paraformaldehyde and embedded in paraffin for subsequent immunohistology.

In vitro study. For primary MGC culture, the procedures were performed as described.4 In brief, 3- to 4-week-old Kun Ming mice were injected i.p. with 10 IU pregnant mare serum gonadotropin and killed 48 h later.63 Superovulated mouse ovaries were harvested and individually transferred into 35-mm Petri dishes containing PBS and then punctured with a syringe to release MGCs from DFs (>200 μm in diameter) under a surgical dissecting microscope. The cell suspensions were plated in

DMEM/F-12 (1 : 1) (Invitrogen, Shanghai, China) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 100 units/ml penicillin plus 100 μg/ml streptomycin (Gibco).

*Cell treatment*After exposure to 200 μM H_2O_2 (Sigma, St. Louis, MO, USA) for 1 h, MGCs were rinsed with PBS and grown in serum-free DMEM/F-12 containing 7.5 IU/ml FSH for 6h (For RT-PCR), 12h (For TUNEL assay). In some experiments, LY294002 (20 μM) was added 1h before FSH treatment.*TUNEL assay*

Terminal deoxynucleotide triphosphate transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) was accomplished using an in situ Cell Death Detection Kit (Roche, Switzerland) to detect cellular apoptosis, After TUNEL reactions, cell climbing sheets or ovarian sections were mounted with VECTASHIELD Mounting Medium and examined under a laser-scanning confocal microscope (Carl Zeiss)

Determination of Bim, caspase-3, Puma mRNA levels by RT-PCR

Total RNA was isolated from the treated GCs using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The quantity and purity of the RNA were determined by measuring the ratio of absorbance at 260/280 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). The total RNA (1 μg) was then reverse transcribed by adding 5×gDNA Eraser Buffer and gDNA Eraser, and incubating at 42 °C for 2 min. PrimeScript RT Enzyme Mix I, RT Primer Mix and 5×PrimeScript Buffer were subsequently added, and the mixture was incubated at 37 °C for 10 min. The samples were then heated at 85 °C for 5 s to inactivate the enzymes, and stored at 20 °C. A Rotor Gene-3000 PCR machine (Gene Co., Hong Kong) and a real-time PCR kit (SYBR® Premix Ex TaqTM II) were used according to the manufacturer's instructions. The primers used for the target genes and β-Actin (synthesized by Shanghai Generay Biotech Co., Ltd) are shown in Table 1. *Statistical analysis*

Data are presented as the means ± SEM. Data were tested for normal distribution, and statistical significance was assessed by the independent sample t-test using SPSS version 11.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were considered statistically significant if $P < 0.05$.

Results

FSH protected GCs from apoptosis in vivo

As shown in Fig. 1, the 3NP-treated group had significantly increased apoptosis rate compared to the control group ($P < 0.05$). Compared with the 3NP-treated group, the FSH-treated group had significantly decreased apoptosis rate ($P < 0.05$). Compared with the FSH-treated group, 3NP treatment significantly inhibited the decrease of apoptosis rate ($P < 0.05$).

FSH reduced PUMA expression in GCs

As shown in Fig. 2, the 3NP-treated group had significantly increased the mRNA levels of puma compared to the control group ($P < 0.05$). Compared with the 3NP-treated group, the FSH-treated group had significantly decreased the mRNA levels of bim, caspase-3 and puma ($P < 0.05$). Compared with the FSH-treated group, 3NP treatment significantly inhibited the decrease of puma mRNA levels ($P < 0.05$).

FSH protected GCs from apoptosis in vitro

The H2O2-treated group had significantly increased apoptosis rate compared to the control group ($P < 0.05$). Compared with the H2O2-treated group, the FSH-treated group had significantly decreased apoptosis rate ($P < 0.05$). Compared with the FSH-treated group, H2O2 treatment significantly inhibited the decrease of apoptosis rate ($P < 0.05$) (Fig 3). *FSH attenuated PUMA expression in GCs*

As shown in Fig. 4, the H2O2-treated group had significantly increased the mRNA levels of puma compared to the control group ($P < 0.05$). Compared with the H2O2-treated group, the FSH-treated group had significantly decreased the mRNA levels of Bim, caspase-3, puma ($P < 0.05$). Compared with the FSH-treated group, H2O2 treatment significantly inhibited the decrease of puma mRNA levels ($P < 0.05$).

FSH reduced PUMA expression in a PI3K-AKT-dependent manner

As shown in Fig. 5, the H2O2-treated group had significantly increased the mRNA levels of Puma compared to the control group ($P < 0.05$). Compared with the H2O2-treated group, the H2O2/FSH-treated group had significantly decreased the mRNA levels of Bim, caspase-3 and puma ($P < 0.05$). Compared with the H2O2/FSH-treated group, LY294002 (PI3K inhibitor) treatment significantly inhibited the decrease of puma mRNA levels ($P < 0.05$).

Discussion

The demand for oxygen increases during follicular development, which can easily shift the redox balance of GCs and increase the ROS content, causing subsequent apoptosis and follicular atresia (Agarwal *et al*. 2005). Therefore, identifying the factors that inhibit the oxidative stress and apoptosis in ovarian GCs will serve to enrich our understanding of follicular development and have a great impact on our practice. Ovarian cell apoptosis can lead to follicular atresia and luteal dissolution, and in the process of cell apoptosis, it is regulated by a series of proteins related to apoptosis, genes and expression products, such as the anti-apoptotic protein Bcl-2 family and the homologous pro-apoptotic protein Bax, which play an important role in the process of apoptosis (Šedová *et al*. 2018). In particular, we investigated the role of FSH and PUMA in GCs apoptotic processes related to follicular atresia in mouse ovaries under in vitro as well as in vivo conditions. Taken together, our results demonstrated that FSH could downregulate the PUMA expression induced by oxidative stress in vivo as well as in vitro.

Our previous study demonstrated that ROS can induce PUMA expression in GCs, and showed that one of the upstream PUMA is FOXO1 (Liu *et al*. 2015). Here we tried to demonstrate weather there are any factors that are responsible for inhibiting the ROS induced apoptosis in GCs, and which pathway the factors follow to downregulate the expression of PUMA. Gonadotrophins (FSH and luteinizinghormone) are primarily responsible for the selection of dominant follicles (Mihm *et al*. 2008, Popelová *et a*l. 2018). FSH could promote antral follicle growth and development into pre-ovulatory follicles, which will maintain anovulation without stimulation by luteinizinghormone (Maillet *et al*. 2005). FSH withdrawal (coasting) during this stage leads to GCs apoptosis and follicular atresia.

Two major cascades lead to apoptosis: the extrinsic pathway (type I cell death pathway), which activates cell surface receptors in response to external signals such as fas ligand, and the intrinsic pathway (type II cell death pathway), which causes cytochrome c release into the cytosol following mitochondrial membrane disruption that is induced by Bcl-2 family members. Previous studies identified Bim as a BH3-only protein that promotes apoptosis by changing the balance between pro- and anti-apoptotic members of the Bcl-2 family, thereby affecting permeability of the

mitochondrial membrane to cytochrome c, which further triggers the activation of caspase-9, caspase-3 and the cell death program (Shen *et al*. 2014). GCs apoptosis driven by FSH withdrawal induced hallmarks of mitochondrial apoptosis (Bim, caspase-9 and caspase-3), which were then suppressed in the presence of FSH. In this study, we found that the FSH-treated group had significantly decreased the mRNA levels of bim, caspase-3 and puma compared to the 3NP-treated group in vivo. 3NP significantly induced cells apoptosis; however, FSH treatment significantly decreased the apoptosis rate in 3NP-induced GCs. This result indicated that FSH can protect against 3NP-induced apoptosis in GCs.

Previously, we found that oxidative stress induced by 3NP injection in mice increases PUMA mRNA expression. In consistence with the in vivo experiment, our in vitro experiment showed that FSH could downregulate the PUMA mRNA expression. However, the level of PUMA expression in FSH treated GCs with 3NP treatment was still higher than that of in control GCs. As many BH3-only Bcl-2 subfamily proteins have similar effects on cellular apoptosis, it is possible that oxidative stress-induced apoptotic signaling was regulated by other BH3-only domain proteins after FSH treatment.

To further validate our hypothesis, that FSH could inhibit the PUMA expression to downregulate apoptosis induced by ROS. In this study, we found that H2O2 significantly induced cells apoptosis in vitro; however, FSH treatment significantly decreased the apoptosis rate in H2O2-induced GCs. This result indicated that FSH can protect against H2O2-induced apoptosis in GCs.

Moreover, the result demonstrated that FSH could downregulate the PUMA expression at mRNA level, through in vivo and in vitro experiments; we showed that FSH could downregulate the PUMA expression at mRNA level to inhibit the apoptosis induced by H2O2.

The above experiments indicate that FSH could regulate the PUMA expression to inhibit the oxidative stress induced apoptosis, FSH regulates the growth and differentiation of follicular GCs through several downstream signaling pathways, including PKA, PI3K, AKT p38-MAPK and ERK1/2 although their interactions in response to FSH remain to be investigated (Hunzicker-Dunn *et al*. 2006). Our lab had

previously demonstrated that FSH could regulate the FOXO expression to inhibit the oxidative stress induced apoptosis (Liu *et al*. 2015). We demonstrated that LY294002 (PI3K inhibitor) abolished the downregulation of PUMA mRNA by FSH in vitro. Therefore, our data raised the possibility that FSH prevents PUMA expression from undergoing apoptosis in a PI3K/AKT-dependent manner.

In conclusion, we demonstrated that FSH could downregulate PUMA mRNA expression to inhibit the ROS induced apoptosis in vivo and in vitro experiments and this process is through PI3K/AKT pathway.

Competing interests

There is no conflict of interest.

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Table 1. Primer sequences used for qRT-PCR analysis of target genes.

Target genes	Primer sequences (5'-3')
PUMA	ATGGCGGACGACCTCAAC
	AGTCCCATGAAGAGATTGTACATGAC
Bim	TATGGAGAAGGCATTGAC
	TGTGGTGATGAACAGAGG
Caspase-3	ACAGCACCTGGTTACTATTC
	CAGTTCTTTCGTGAGCAT
β-Actin	GCTGTCCCTGTATGCCTCT
	GTCTTTACGGATGTCAACG

Figure Legends

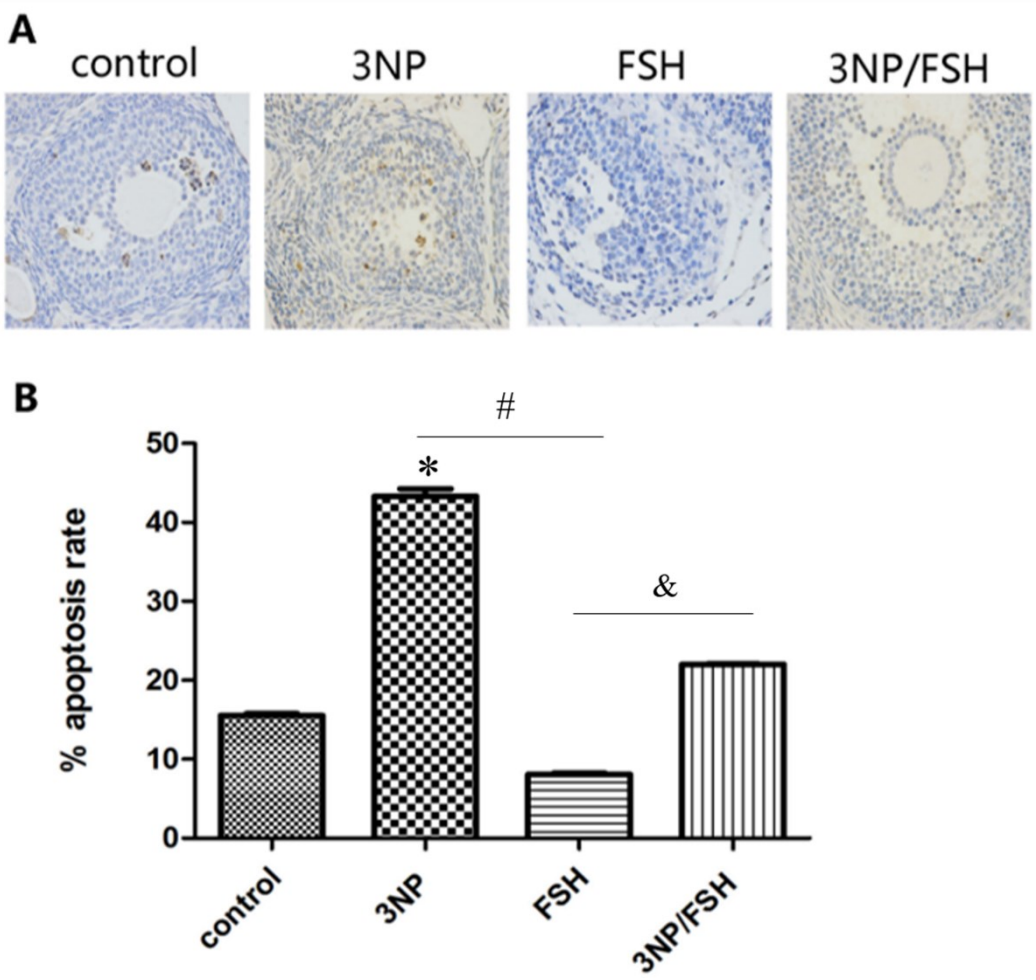

Figure 1. FSH protected MGCs from apoptosis *in vivo*.

Data are presented as the means ± SEM, n = 3. * $P < 0.05$ compared with control group; # $P < 0.05$ compared to the 3NP-treated group; & $P < 0.05$ compared to the FSH-treated group.

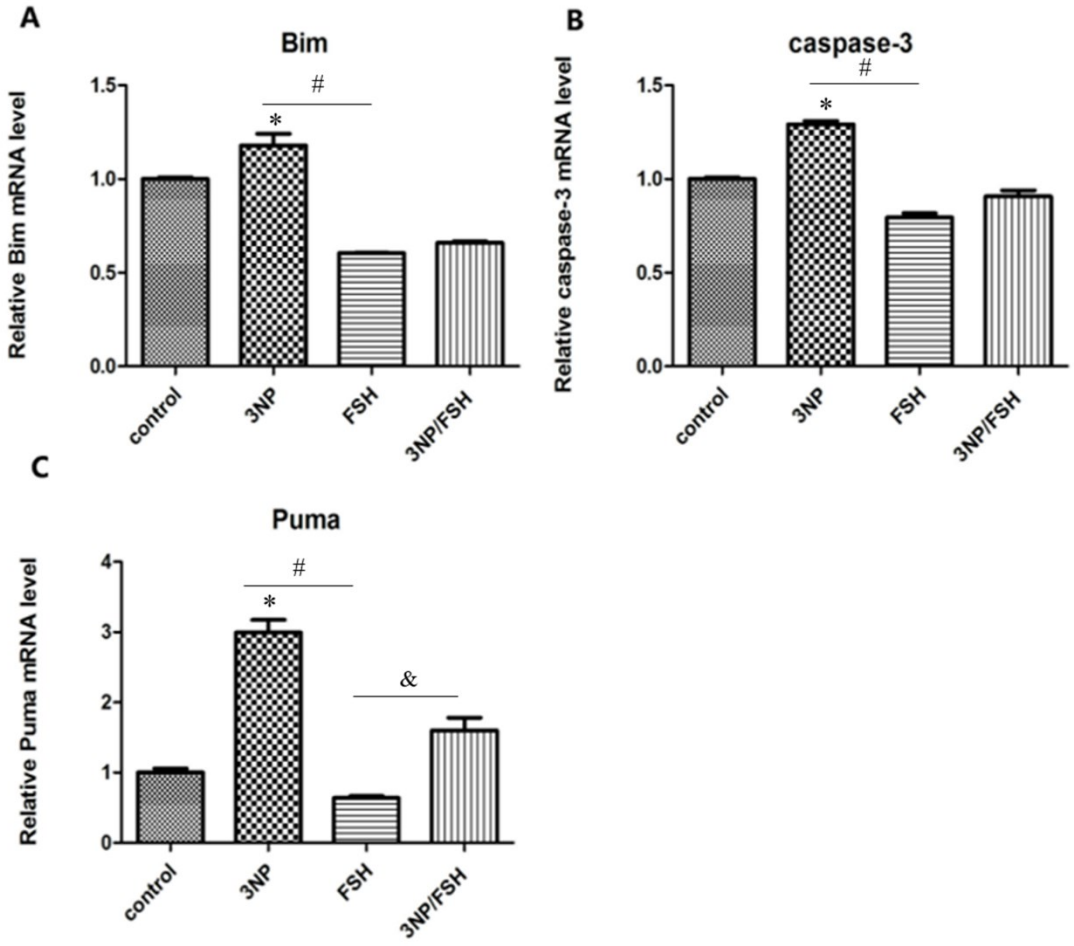

Figure 2. FSH reduced PUMA expression in ovarian granulosa cell.

(A) mRNA levels of Bim. (B) mRNA levels of caspase-3. (C) mRNA levels of Puma. Data are presented as the means ± SEM, n = 3. * $P < 0.05$ compared with control group; # $P < 0.05$ compared to the 3NP-treated group; & $P < 0.05$ compared to the FSH-treated group.

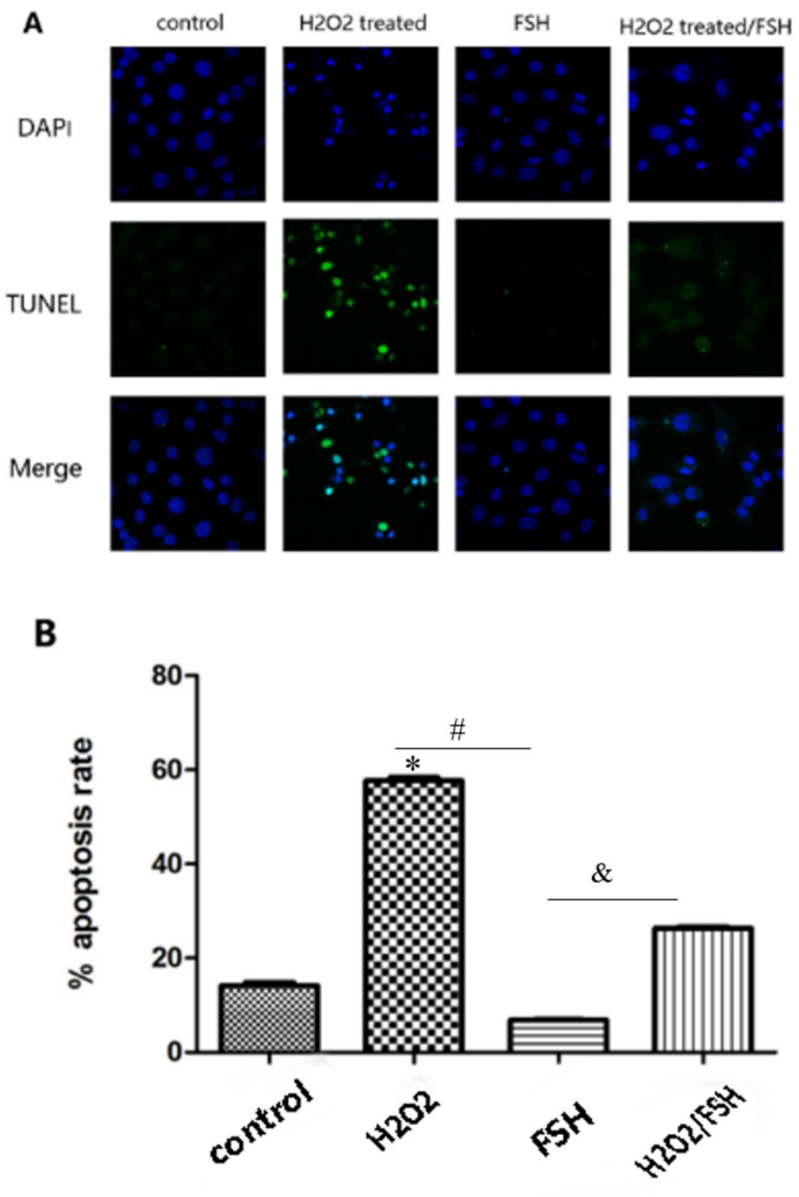

Figure 3. FSH protected granulosa cell from apoptosis in vivo.

Data are presented as the means ± SEM, n = 3. * $P < 0.05$ compared with control group; # $P < 0.05$ compared to the H2O2-treated group; & $P < 0.05$ compared to the FSH-treated group.

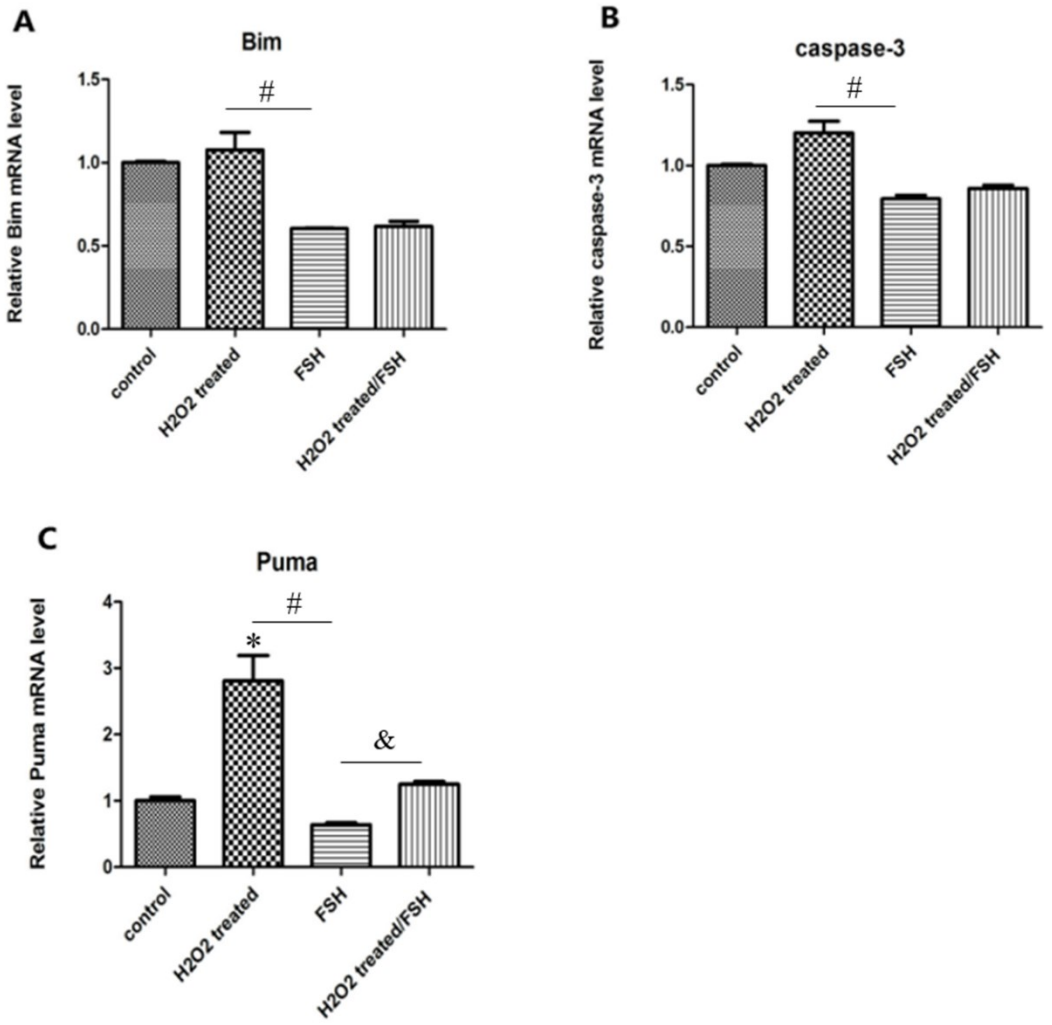

Figure 4. FSH attenuated PUMA expression in granulosa cell**.**

(A) mRNA levels of Bim. (B) mRNA levels of caspase-3. (C) mRNA levels of Puma. Data are presented as the means ± SEM, n = 3. * $P < 0.05$ compared with control group; # $P < 0.05$ compared to the H2O2-treated group; & $P < 0.05$ compared to the FSH-treated group.

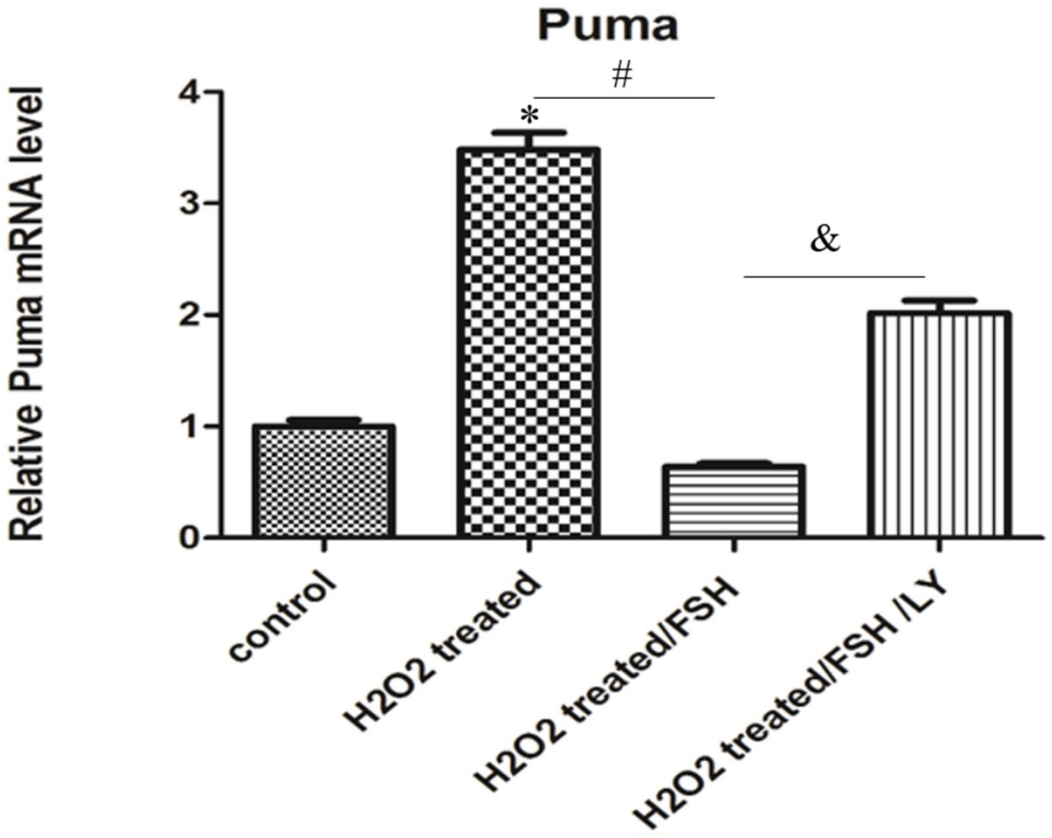

Figure 5. FSH reduced PUMA expression in a PI3K-AKT-dependent manner. Data are presented as the means ± SEM, n = 3. * $P < 0.05$ compared with control group; # $P < 0.05$ compared to the H2O2-treated group; & $P < 0.05$ compared to the H2O2/FSH/LY-treated group.