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The Effect of FSH-Induced Nuclear Exclusion of FOXO3/4 on Granulosa Cell Proliferation and Apoptosis of Hen Ovarian Follicles

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Abstract: Background: Follicle stimulating hormone (FSH) is key regulator for follicular development, differentiation and maturation, the effects involve various intra follicular factors, such as members of FOXO subfamily. However, the specific role and mechanism of FOXO3 and FOXO4 in growth and development of hen follicles by affecting granulosa cell (GC) division and FSH response function are still unclear. **Method:** This study selected GCs from 6-8mm chicken follicles to detect FSH induced phosphorylation and nuclear rejection of FOXO3/4, and its regulatory effects on follicular GC proliferation, differentiation and apoptosis, using immunofluorescence, Western blotting, RT-qPCR and flow cytometry methods. **Results:** This study found that the expression level of FSHR mRNA was highest in GC treated with 10 ng/ml FSH for 8 and 12 hours. The level of p-FOXO3/4 protein significantly increased in cells treated with FSH for 12 hours, while the expression level of non phosphorylated FOXO3/4 significantly decreased. After co-treatment with 10ng/ml LMB, FOXO3/4 phosphorylation was effectively prevented. The immunofluorescence results showed that FOXO3 and FOXO4 were originally distributed in GC nucleus and cytoplasm, whereas they were almost accumulated in cytoplasm as treated with FSH for 12h. Conversely, FOXO3/4 nuclear translocation was blocked by LMB. Moreover, RT-qPCR and flow cytometry results showed that FSH treatment significantly increased proliferation and differentiation of cell, but significantly reduced GCs apoptosis. However, LMB also eliminated these stimulating or inhibitory effects on cell proliferation. **Conclusion:** These findings provided new evidence that FSH induced FOXO3/4 nuclear rejection promoted GCs proliferation and reduced GCs apoptosis during hen follicular development.

Keywords: chicken; FOXO3/4; nuclear exclusion; cell proliferation; granulosa cell

1. Introduction

Ovarian follicular development plays a pivotal role in poultry reproduction, affecting clutch traits and thus egg production [1], it primarily depends upon proliferation, differentiation and apoptosis of granulosa cells (GCs) and theca cells as well as oocyte growth and mature of hen ovary [2–4]. Among them, the GCs not only nurture the developing oocytes in follicles, but also play an essential role in folliculogenesis, follicle selection and a well-organized preovulatory hierarchy by governing steroidogenesis and release of the intra-ovarain factors, such as the steroidogenic acute regulatory protein (StAR) whose main function is to transport cholesterol from the outer membrane to the inner membrane of mitochondria for the synthesis and secretion of progesterone (P4) in granulosa cells of follicles [5]; cytochrome P450 family 11 subfamilies A member 1 (CYP11A1/P450scc) that plays a catalytic role in the hydrolysis of cholesterol to produce progesterone, is the rate-limiting enzymatic step within granulosa cells of the hen ovary [6,7];

Myelocytomatosis oncogene (c-MYC) is a well-known nuclear oncoprotein having multiple functions in cell proliferation, apoptosis and cellular transformation of several developing organs [8,9]; proliferating cell nuclear antigen (PCNA) is a central factor in cell cycle regulation and DNA replication, and shown to have a fundamental role in cellular proliferation [10,11]; B-cell lymphoma-2 (BCL-2) mainly serves as one anti-apoptotic member of the family by sequestering proforma of death-driving cysteine proteases [12], and the caspase-3 (CASP-3) acts as the main executioner of apoptosis in multicellular organisms and also in diverse biological processes, including cell differentiation, and proliferation, which is under the tight control of the BCL-2 family proteins [1,13]. More importantly, various members of gonadotropins and their receptors, such as follicle-stimulating hormone (FSH) and FSH receptor (FSHR) are also implicated in GC differentiation, maturation and steroidogenesis, and follicle development and selection [14–16].

FSH is a 35.5 kDa heterodimeric glycoprotein composed of two polypeptide units including alpha and beta subunits being synthesized and secreted by the anterior pituitary gonadotropic cells, acts as the primary hormonal inducer of ovarian follicle maturation and a critically important regulator of steroidogenesis in granulosa cells [17,18]. Its biological functions are activated through the interaction with its specific FSHR receptor in poultry [19]. FSH has been demonstrated to induce follicle selection marked by the highest expression levels of *FSHR* mRNA within the granulosa layer from individual prehierarchical follicles of 6-8 mm in diameter [15], and affect granulosa cells proliferation and differentiation which synthesize progesterone and estrogen crucial for the growth and maturation of preovulatory follicles promoted by the up-regulation of StAR, CYP11A1, and steroid 17-alpha-hydroxylase (CYP17) expression in hen ovary [3,20]. The function and regulatory mechanism of FSH in ovarian follicle growth, differentiation and maturation by its interaction with FSHR to activate a number of downstream effectors and intracellular signals have been well documented [2,21–24], but characterization of *FSHR* expression in the GCs of ovarian follicles induced by FSH remain to be further clarified. Furthermore, a wide variety of transcription factors, such as the members of forkhead box O (FOXO) subfamily were proved to be involved in ovarian follicle development and ovarian function by response to FSH signals [25–27], and the FOXO members mainly function to promote GC apoptosis and follicular atresia [25,28,29]. However, FSH plays the opposite role by facilitating GC proliferation and follicle growth, selection, and maturation [3,20,30,31]. Therefore, the contribution of the FOXO members to FSH signaling cascades has been attracting more and more researcher`s attention.

The members of FOXO family, including FOXO1 (also known as FOXO1A), FOXO3, FOXO4 and FOXO6 regulate many cellular programs, including cell proliferation, apoptosis, cell cycle transitions and DNA repair, autophagy, cell metabolism and responses to oxidative stress, which are of key importance in the decision over cell fate [32–34]. In mammalian ovaries, FOXO1 and FOXO3 were found to regulate follicular atresia by promoting GCs apoptosis [25,35]. Although the FSHR signals primarily via the cAMP/protein kinase A pathway [3,36], the previous studies showed that the phosphatidylinositol-3 kinase (PI3K)/3-PhosPhoinositide-dePendentProteinkinase-1 (PDK1) pathway is also activated by FSH in rat/mouse GCs, and the AKT activated downstream of PI3K in FSH-treated cells then phosphorylates FOXO1 to abrogate its transcriptional activity [34,35]. The FOXO3 is a proapoptotic molecule, and the expression levels of *FOXO3* mRNA in GCs of porcine ovaries increased during atresia, while FOXO3 protein was abundant in granulosa cells of early atretic follicles [25]. In addition, the GC apoptosis was significantly and positively correlated with the total FoxO3 and negatively correlated with the phosphorylated FOXO3 protein (p-FOXO3) levels in the polycystic ovary syndrome (PCOS) patients [29]. Furthermore, the recent study has revealed that FOXO3 was expressed in the follicles at all stages, and the expression level in follicles increased with increasing follicle hierarchy, and promotes GC apoptosis of hen ovarian follicles [27].

Similar to other members of FOXOs, FOXO4 has been proved to play major roles in control of cellular proliferation, cell cycle arrest and apoptosis in mammals [37–39]. And the FOXO4 functions in a mechanism of cellular nuclear translocation and transcriptional regulation alike with its isoform FOXO3 factor although their molecular component and structure are divergent from each other

[40,41]. FOXO4 can be phosphorylated by PI3K/PDK1 signaling on its two phosphorylation motif sites (Thr-32 and Ser-197) resulting in its inactivation and nuclear exclusion [42,43]. In the absence of PI3K/PDK1 activation, FOXO4 is located in the nucleus, where it functions as a transcription factor [44]. And in the presence of low levels of oxidative stress generated by treatment of cells with H2O2, FoxO4 becomes activated and remains in the nucleus. This effect appears to involve phosphorylation of FOXO4 by jun N-terminal kinase (JNK) at the two sites (thr- 447 and thr- 451) [45]. In addition, the monoubiquitylation on two conserved lysine residues (K199 and K211) of FOXO4 in response to increased cellular oxidative stress facilitates its re-localization to the nucleus and an increase in its transcriptional activity [46]. Recently, it has been revealed that the expression of FOXO4 is tightly controlled by microRNAs, and its subcellular localization is dependent on phosphorylation modification [37]. Thus, the nuclear re-localizations and functions of FOXO4 are firmly commanded by different signaling pathways that depended on the various physiological states or cellular processes. However, the exact roles and mechanisms of FOXO3 and FOXO4 (herein-after abbreviated as FOXO3/4) in prehierarchical follicular growth and development by controlling the GC proliferation and apoptosis under the FSH signals of chicken ovaries remain unclear.

In this study, we provide evidence to explain and support the claim that FSH induces phosphorylation and nuclear exclusion of the FOXO3/4 in granulosa cells of hen ovarian follicles. The FSH-induced nuclear exclusion of FOXO3/4 leads to the inhibition of cell apoptosis but increased proliferation and differentiation of granulosa cells. To the best of our knowledge, this study for the first time presents the novel data on the nuclear exclusion of FOXOs by involvement of FSH in hen ovarian granulosa cells. The current finding has laid a dependable foundation for further understanding the molecular regulatory mechanism of FOXO3/4 nuclear exclusion in follicular development and atresia of chicken ovary.

2. Materials and Methods

2.1. Ethics Statement

In this study, all procedures conducted in chickens were approved by the Institutional Animal Care and Use Committee (IACUC) of Jilin Agricultural University (Changchun, China). The animal experiment was performed in compliance with the ARRIVE guidelines [47]. Chickens were sacrificed before removing organs following the IACUC guidelines for experimental animals (Permission No. GR (J) 19-030). Euthanasia of the hens is fully compliant with the Chinese applicable laws and regulations concerning the care and use of laboratory animals, which was issued based on the Regulations for the Administration Affairs Concerning Experimental Animals of the State Council of the People's Republic of China (2017 Revision). All of our efforts have been made to minimize the suffering of the animals.

2.2. Sampling and Granulosa Cell Culture

A commercial strain of Lohmann Brown laying hens was raised in laying batteries according to the standard husbandry practices as previously reported [48]. 22 hens were procured from the population and euthanized at 21 weeks of age, and the follicles of 6-8mm diameter size were immediately collected. Follicle GCs were subsequently isolated from the follicles and cultured according to a protocol that was previously reported by us [4]. A cell suspension was then prepared from the isolated GCs before adding M199 culture medium (Biosharp Life Sciences, Anhui, China) containing 10% of newborn calf serum, 100 IU/ml of penicillin, 75 IU/ml of streptomycin, and 10 ng/ml of insulin. The GCs was then incubated in a 5% CO_2 and 95% air at 37℃. Cells were treated with 10 ng/ml FSH (R&D Systems, Shanghai, China) for 0h, 8h, 12h, and 24h, and then expression levels of FSHR gene were quantified. To evaluate expression of the genes (such as FOXO3/4 et al.) directly induced by FSH, cells were cultured with or without 10 ng/ml FSH for 12 h in the presence or absence of 10 ng/ml Leptomycin B (LMB; Beyotime, Shanghai, China).

2.3. Immunofluorescence Staining

In the process of cell culture, six-well tissue culture plates were filled with circular glass coverslips, which were placed there to promote cell growth. Following this, cells were immobilized by use of 4% paraformaldehyde (PFA, Beyotime, Shanghai, China), and the ends of the coverslips were homogeneously coated with an immunostaining guard pen (Biosharp Life Sciences, Anhui, China) to generate a 1mm oily barrier. The cells were then incubated with primary antibodies: anti-FOXO3 (Proteintech, Wuhan, China, 1:500 dilution), anti-FOXO4 (Bioss, Beijing, China, 1:500 dilution) at 4°C for 12 hours. After washing with PBS, the cells were incubated with fluorescent-conjugated goat anti-rabbit IgG antibody (Epizyme Biotech, Shanghai, China, 1:1000 dilution). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Epizyme Biotech, Shanghai, China). All immunofluorescence images were captured by laser confocal microscope (Zeiss).

2.4. Western Blot Analysis

The procedure made use of a RIPA lysis buffer (Biosharp Life Sciences, Anhui, China) to treat the cells for purposes of protein extraction from the samples. This was then proceeded by the adding of protease inhibitors cocktail (Epizyme Biotech, Shanghai, China) and phosphatase inhibitor cocktail (Epizyme Biotech, Shanghai, China), with subsequent sonication for fragmentation. The concentration of the protein was then adjusted following the instructions provided in the BCA protein assay kit (Beyotime, Shanghai, China). Nitrocellulose membrane transfer was then completed after electrophoresis at 150V in a 10% SDS PAGE gel (Epizyme Biotech, Shanghai, China). After blocking with 5% nonfat milk, the membranes were incubated to the primary antibodies: anti-FOXO3 (Proteintech, Wuhan, China, 1:2000 dilution) and p-FOXO3 (Bioss, Beijing, China, 1:1000 dilution), anti-FOXO4 (Bioss, Beijing, China, 1:1000 dilution) and p-FOXO4 (Bioss, Beijing, China, 1:1000 dilution), and anti-β-actin (Boster Biological Technology, Wuhan, China, 1:10000 dilution) at 4℃ overnight. Then washing with PBS, and the samples were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody as secondary antibody for 1.5 hours. Images were taken and visualized by an ECL Plus western blotting detection system according to the manufacturer's instructions.

2.5. Quantitative Real-Time RT-PCR

To assess the mRNA expression of the target genes in the GCs, real-time quantitative reverse transcriptase PCR (qRT-PCR) was conducted according to our previously described method [48]. The used in this study were shown in Table 1. The reaction system contained SYBR 10 μl, 0.4 μl upper- and 0.4 μl lower-primer, and cDNA 2 μl. The amplification conditions used were 95℃ for 10 minutes and 95℃ for 15 seconds with an annealing temperature of 60℃ for 60 seconds and a total of 40 cycles. The 18S rRNA was used as a normalizing gene.

Table 1. Primer pairs designed for quantitative real-time PCR analysis.

Gene	Forward primer（5'-3'）	Reverse primer（5'-3'）	Accession No.	Size
FSHR	ATGTCTCCGGCAAAGCAAGA	AACGACTTCGTTGCACAAGC	NM_205079.1	147 bp
CASP-3	ATTGAAGCAGACAGTGGACCAGATG	TGCGTTCCTCCAGGAGTAGTAGC	NM_204725.2	111 bp
PCNA	CTGAGGCGTGCTGGG	ATGGCGATGTTGCGG	NM_204170.3	133 bp
StAR	AGCAGATGGGCGACTGGAAC	GGGAGCACCGAACACTCACAA	NM_204686.2	147 bp

CYP11 A1	CCGCTTTGCCTTGGAGTCTGTG	ATGAGGGTGACGGCGTCGATGAA	NM_001001756.1	111 bp
c-MYC	GAGAACGACAAGAGGCGAAC	CGCCTCAACTGCTCTTTCTC	NM_001030952.2	211 bp
BCL-2	CGCTACCAGAGGGAC	GAAGAAGGCGACGAT	NM_205339.3	135 bp
PDK1	AGACATCCCGAGCTACACCT	CGCCTTGGAAGTATTGTGCG	NM_001031352.4	81bp
SGK3	TGCGTCCAGGAATCAGTCTCAC	AAGTCTGCTTTGCCGATCTTTCTC	NM_001030940.2	74bp
18s rRNA	TAGTTGGTGGAGCGATTTGTCT	CGGACATCTAAGGGCATCACA	AF173612.1	169 bp

2.6. Flow Cytometric Analysis

The cells processed as explained earlier were digested with trypsin without EDTA and the cells collected. After centrifugation, the supernatant was ridden, and the cells were washed with PBS, suspended again, and re-centrifuged to eradicate the supernatant. Annexin V/PI double staining was used to detect the level of apoptosis of granulosa cells in different treatment groups. The prepared cell samples were stained according to the instructions of the annexin V-FITC/PI apoptosis test kit which was instantly analyzed using the CytoFlex flow cytometer. In accordance to the scatter plot of the bivariate flow cystoscope, the proliferating live cells were displayed as (FITC-/PI-) in the lower left quadrant. Necrotic cells, or non-viable cells, were displayed in the upper right quadrant as (FITC+/PI+). Some believe these cells to be non-viable apoptotic cells, or dead cells, which are represented as (FITC-/PI+). Apoptotic cells were exhibited in the lower right quadrant as (FITC+/PI-).

2.7. Statistical Analysis

Statistical analysis was implemented d using the SPSS12.0 software package (Sun et al., 2021). All the experiments were repeated at least three times using different batches of sampled birds. The data were analyzed with a one-way ANOVA and Tukey's multiple-comparison test when more than two groups were involved or using a Student's t-test when treatment and control groups were compared after confirming normal distributions for parametric analysis. $P < 0.01$ or $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. The Expression of FSHR mRNA in the GCs Regulated by FSH

To reveal the effects of FSH signaling on the expression and phosphorylation of FOXO3/4 in the undifferentiated GCs by its interaction with the FSHR in the hen ovaries, the expression levels of *FSHR* mRNA in the GCs induced by FSH were firstly determined in the prehierarchical follicles of 6-8 mm diameter. As shown in Figure 1, expression levels of *FSHR* mRNA were dramatically elevated in the GCs cultured with 10ng/ml FSH for for 8h or 12h ($P < 0.01$). However, the levels of *FSHR* mRNA were then markedly declined in the GCs treated with 10ng/ml FSH for 24h ($P < 0.05$) although the *FSHR* mRNA expression levels were significantly higher compared with the controls ($P < 0.05$). Unexpectedly, the *FSHR* mRNA expression levels presented unchanged in the GCs under treatment with 12ng/ml FSH from 0h to 12h compared with the control group ($P > 0.05$). These results provided an evidence for FSH signaling to activate its FSHR pathway that may be involved in the regulation and function of FOXO family members in chicken ovarian follicle development.
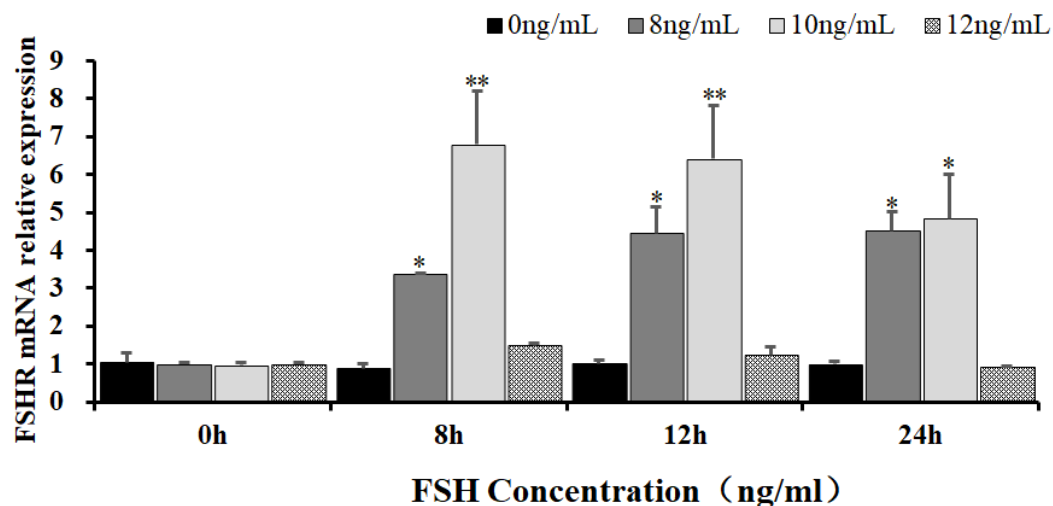
Figure 1. The expression of *FSHR* mRNA in the undifferentiated granulosa cells regulated by FSH. The GCs from the hen ovarian prehierarchical follicles of 6-8mm diameter were cultured at the varied concentrations of 0ng/ml, 8ng/ml, 10ng/ml, and 12ng/ml for 0h, 8h, 12h, and 24h, respectively. The data were presented as the means ± SEM from five replicate experiments. The bars with superscript symbol present the significant difference compared with control groups at **P < 0.01, * *P* < 0.05.

3.2. FoxO3/4 Phosphorylation and Nuclear Exclusion Induced by FSH

In order to assess the actions of FSH on the phosphorylation and nuclear exclusion of the FOXO3/4 in the GCs from the ovarian prehierarchical follicles, the levels of phosphorylated FOXO3/4 (p-FOXO3/4) were analyzed by the western blotting method and their subcellular localizations in the cultured GCs were concomitantly inspected by the immunofluorescence analysis. The results demonstrated that expression levels of FOXO3/4 protein were decreased significantly in the cultured GCs with the treatment of 10ng/ml FSH and co-treatment of 10ng/ml FSH and Leptomycin B (LMB) for 12h compared to the control group ($P < 0.01$, Figure 2A,B). However, the levels of the p-FOXO3/4 were dramatically increased in the GCs under the FSH treatment for 12h and this stimulatory action of FSH was effectively prevented by the co-treatment with 10ng/ml LMB ($P < 0.01$). Moreover, either between FOXO3 and FOXO4 proteins or p-FOXO3 and p-FOXO4 molecules exhibited the greatly similar expression patterns in the GCs with the FSH treatment, respectively. Furthermore, the double immunofluorescence staining revealed that total FOXO3/4 (including FOXO3/4 and p-FOXO3/4) were localized in the GC nucleus and cytoplasm, whereas the nuclear FOXO3/4 were almost failed to be visualized in nuclei but mainly accumulated in the cytoplasm of the GCs treated with FSH for 12h. This indicated that the nuclear FOXO3/4 was re-localized to the cytoplasm of the cells upon induction of FSH signaling. Conversely, this FSH-induced nuclear translocation of the FOXO3/4 in the cultured GCs was greatly blocked by the LMB ($P < 0.01$; Figure 3A,B). These findings revealed that FSH promotes phosphorylation and concomitant nuclear exclusion of FOXO3/4 in the cultured GCs, which may bring directly about changes of the effects of FOXO3/4 on ovarian follicle development.
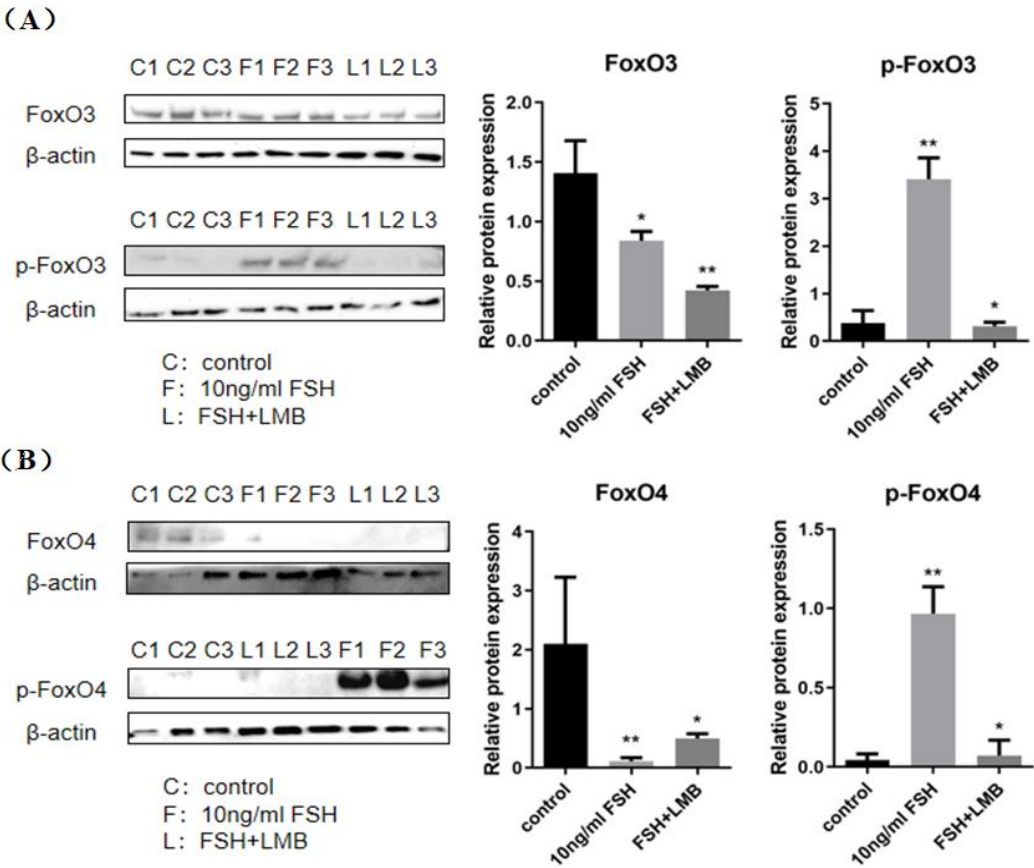
Figure 2. Effects of FSH on the FoxO3/4 phosphorylation in the cultured GCs of the ovarian follicles. (A) The p-FOXO3 levels were determined by western blotting analysis in the GCs treated with or without the 10ng/ml FSH for 12h in the presence or absence of LMB. (B) The p-FOXO4 levels were tested in the GCs under the treatment with or without the 10ng/ml FSH for 12h in the presence or absence of LMB. The data represent the mean ± SEM, (n = 5). Student's t-test, **$P < 0.01$ and *$P < 0.05$.
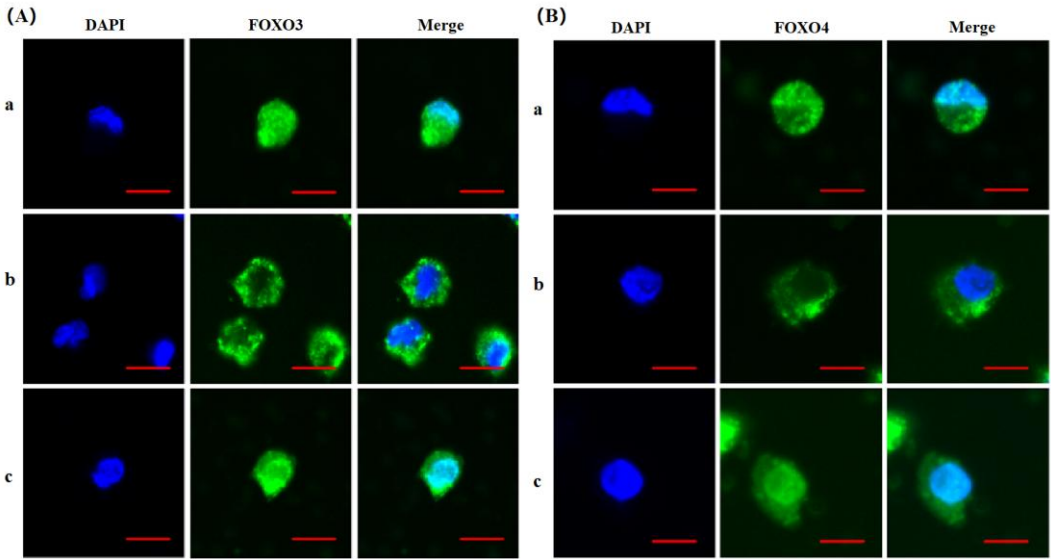

Figure 3. FSH-induced the nuclear exclusion of FOXO3/4 in the cultured GCs of the ovarian follicles. (A) Immunofluorescence detection of FOXO3 subcellular localization in the GCs. The GCs were treated with or without the 10ng/ml FSH for 12h in the presence or absence of LMB, which were stained using goat anti-rabbit FOXO3 (green). The nucleus was stained with DAPI (blue). scale bar = 5μm. (B) Immunofluorescence detection of FOXO4 subcellular localization in the GCs. FOXO4 (green) and nucleus stained with DAPI (blue). a. control,

without treatment of FSH or LMB; b. treatment of the cells with 10ng/ml FSH for 12h; c. co-treatment of the cells with 10ng/ml FSH plus LMB for 12h. n = 3 independent experiments. Leica DMI8, 600X; scale bar = 2.5 μm.

3.3. Expression of the Genes Associated with Cell Proliferation, Differentiation and Apoptosis of the GCs Induced by FSH

To further explore biological functions of the FSH-induced phosphorylation and nuclear exclusion of FoxO3/4 in ovarian follicle development and growth, expression of a battery of candidate genes including *MYC*, *PCNA*, *STAR*, *CYP11A1*, *BCL2*, and *CASP3* that are tightly associated with cell proliferation, differentiation and apoptosis of the GCs were examined. It has now been proved that the mRNA expression levels of the *MYC*, *PCNA* and *BCL2* genes that involved in follicle development by promoting cell proliferation and anti-apoptosis, *STAR* and *CYP11A1* genes that serve as indicators for GC differentiation were dramatically enhanced in the GCs by the treatment of FSH, while the expression level of the *CASP3* mRNA was significantly reduced. However, all of these stimulatory or inhibitory effects induced by FSH on expression of the genes were predominantly abolished by the LMB ($P < 0.01$; Figure 4). These results indicated that the FOXO3/4, critical factors in promoting cell apoptosis oppositely pay the roles in invigorating GCs proliferation and differentiation but reducing apoptosis in response to the FSH-induced phosphorylation and nuclear exclusion during ovarian follicle growth and development.
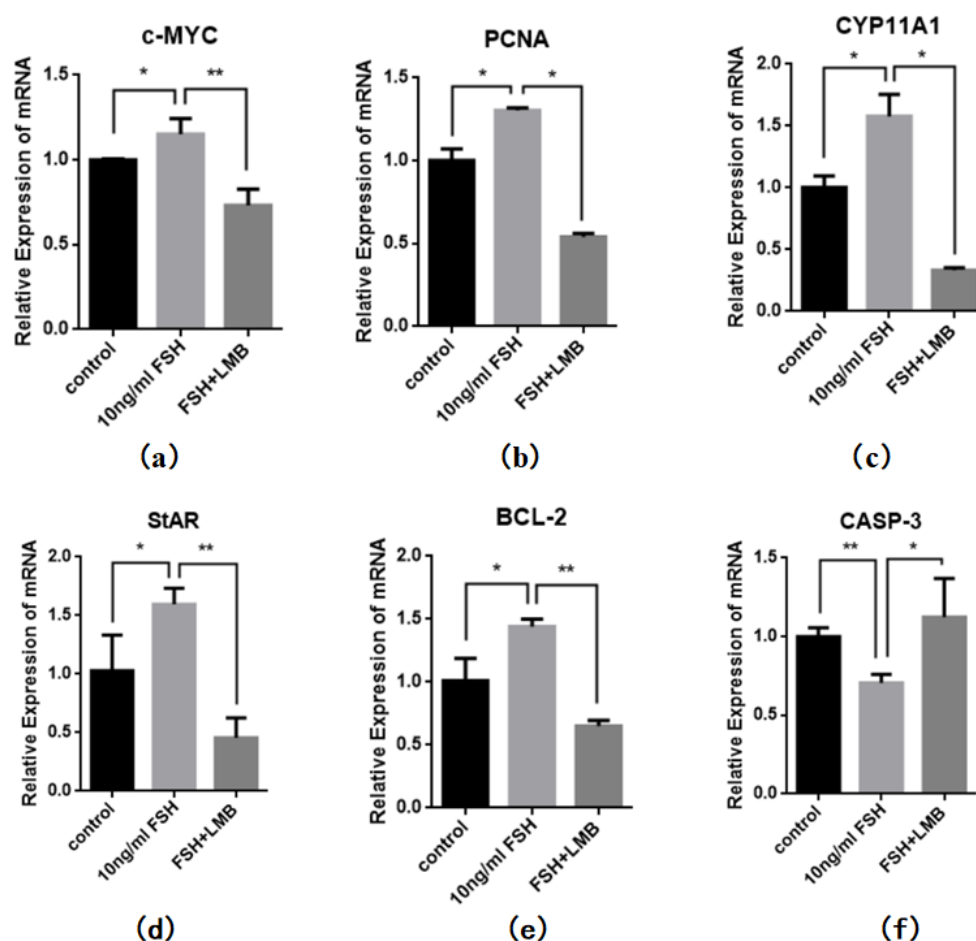

Figure 4. FSH-induced expression of *MYC*, *PCNA*, *StAR*, *CYP11A1*, *BCL-2*, and *CASP-3* genes in the cultured GCs. The data were presented as the means ± SEM from five replicate experiments, and bars with superscript symbol present the significant difference compared with control groups at **$P < 0.01$, * $P < 0.05$.

3.4. Effects of FSH-Induced Phosphorylation and Nuclear Exclusion of FoxO3/4 on GCs Proliferation and Apoptosis

Based upon the molecular roles of the FSH-induced phosphorylation and nuclear exclusion of FoxO3/4 in regulation of the aforementioned genes, flow cytometric analysis was used to scrutinize proliferation and apoptotic rate of the cultured GCs with or without FSH treatment. As shown in Figure 5, a significantly higher cell proliferation rate and lower apoptotic rate of the GCs were observed in the FSH treatment group compared to the control group ($P < 0.05$). Nevertheless, these FSH-induced effects on proliferation and apoptosis of the GCs were significantly abrogated by the LMB ($P < 0.05$). These results solidify the claim that the transcriptional factors FOXO3/4 function to promote GC proliferation and apoptosis via the FSH signaling induced phosphorylation and nuclear exclusion of FOXO3/4 in ovarian follicle growth and development of hens.
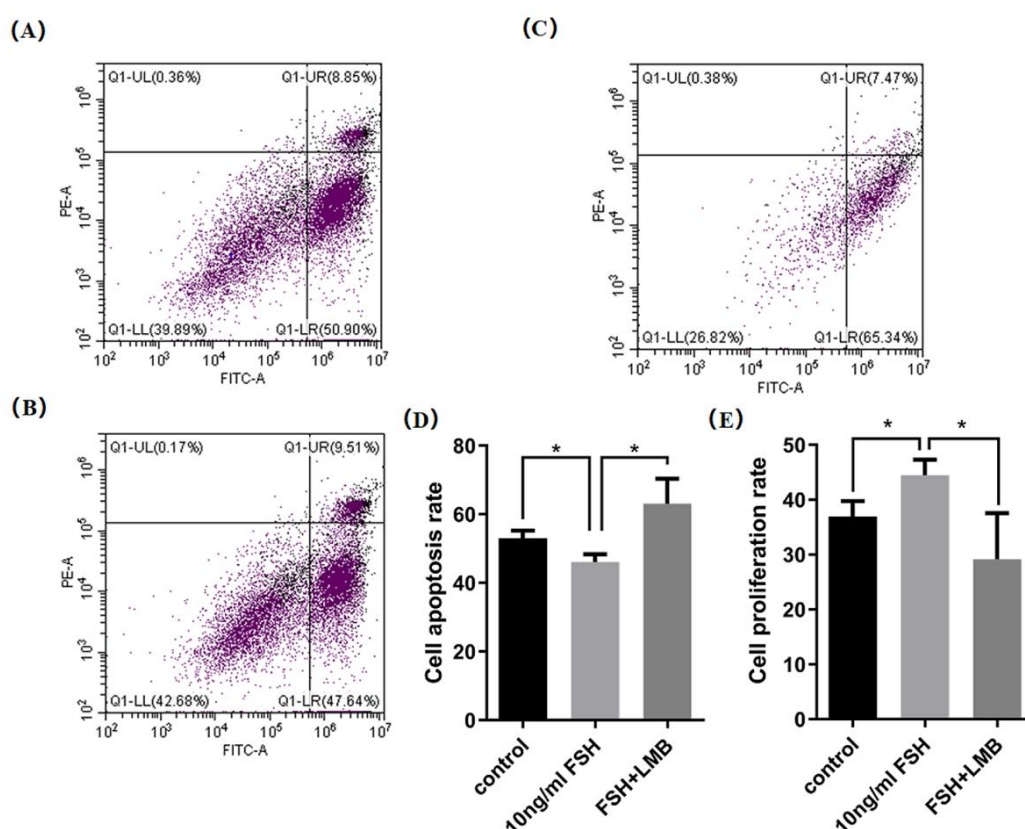

Figure 5. FSH-induced nuclear exclusion of FoxO3/4 on proliferation and cell apoptosis of the cultured GCs. The data were presented as the means ± SEM from three replicate experiments, and bars with superscript symbol present the significant difference compared with control groups at * $P < 0.05$.

4. Discussion

The ovarian follicle growth and development in chicken is a highly intricate and coordinated process involving a multitude of biological events controlled by reproductive hormones in the ovary [48–50], which predominantly contribute to egg productivity and reproductive performance of laying hens [4]. In this process, FSH signaling has a fundamental role in ovarian follicle development, follicle selection, and growth and differentiation (presenting a well-organized preovulatory hierarchy) by interaction with its receptor FSHR, a member of the rhodopsin-like G protein-coupled receptor family, being specifically expressed on ovarian follicular GCs [2,23,51,52], which subsequently activates the cAMP/protein kinase A pathway and indirectly initiates the production of several intra-ovarian factors and endocrine hormones, including CYP11A and STAR expression [21,22,24,53]. Moreover, the higher expression levels of FSHR in the GCs are required for follicle selection and immediately initiate a rapid growth in size of the pre-ovulatory follicles companied by GC

differentiation and steroidogenesis [3,15,54]. However, in the earlier studies it was found that levels of FSHR expression were not responsive to the FSH challenges in the GCs from the ovarian prehierarchal follicles of 6-8mm in diameter following the treatment with FSH for 1h or 3h, and simultaneously the cells failing to initiate a significant increase in cAMP production in vitro [15,55]. The reported results appeared paradox with our current data that levels of *FSHR* mRNA expression were dramatically elevated in the cultured GCs with 10ng/ml FSH treatment for 8h or 12h, and which concomitantly brought about a significant increase of the *STAR* and *CYP11A1* expression as well as a memorable augment of *c-MYC* and *PCNA* expression in the GCs and cell proliferation in the hen ovarian follicles. These present results corroborate the previous findings that the *FSHR* expression levels were remarkably elevated in the GCs of ovarian follicles challenged by FSH in chicken, human and mammals in vitro [27,56–59]. Additionally, Upon FSH stimulation, the notably raised *FSHR* expression levels were examined in the human embryonic kidney (HEK) 293 cells. *FSHR* expression in the CHO-cell system, as detected by FSH binding to receptors, was approximately threefold greater than expression in primary granulosa cells. Therefore, these studies have provided convincing evidence that in response to FSH stimulation, *FSHR* expression is significantly up-regulated in the GCs from the ovarian follicles of 6-8mm in diameter, which corresponds to that a higher level of *FSHR* expression in the GCs, as aforementioned, is required for initiating the rapid follicle growth, differentiation and steroidogenesis of the hen ovary [3,54].

The FOXO family members as the crucial downstream regulators of FSH-induced signaling pathways, including FSHR/PI3K/AKT path, have recently been highlighted as an essential transcriptional factors of the important effectors involved in ovarian follicle growth, maturation and atresia by modulating the GC proliferation, differentiation and apoptosis [60–63]. Previous studies have well documented that FOXO3/4 belong to the FOXO subfamily of transcription factors and have several biological functions by regulating expression of genes associated GC proliferation, apoptosis, cell cycle arrest, autophagy and oxidative stress resistance [25,39,43,64]. In comparison, there is limited information on the regulatory mechanism and their roles in chicken ovarian GC proliferation, differentiation, and apoptosis, particularly, of which was induced by FSH signaling [61]. Prior study revealed the FOXO3 can be negatively regulated by its phosphorylation via the PI3K/Akt signaling pathway and ultimately drives apoptosis when activated in mammals [27]. In this study, we have demonstrated that the expression levels of FOXO3/4 were significantly decreased in the cultured GCs with stimulation of FSH; conversely levels of the p-FOXO3/4 were dramatically elevated in the cells. Whereby, the increased phosphorylation levels of FOXO3/4 were likely mainly caused by the activation of PI3K/AKT signaling under the stimulation of FSH as the previously reported [34,35,42]. Furthermore, relevant studies have shown that apoptosis was significantly and positively correlated with the total FoxO3 and negatively correlated with the p-FoxO3 protein levels in PCOS patients [29], and the FOXO3 is expressed in ovarian follicles and GCs, and promotes apoptosis of hen ovarian GCs [27]. Considering the results currently identified in this study, of which a memorable increase in cell proliferation but a remarkable decrement in apoptosis of the GCs were determined in the GCs by the treatment of FSH, accordingly, it was suggested that the FSH-induced phosphorylation of FOXO3/4 may facilitate the ovarian follicle growth and prevent follicular atresia by attenuating GC apoptosis of the chicken ovary.

To further investigate the exact roles and molecular mechanism of the p-FOXO3/4 in controlling the ovarian GC apoptosis, the FSH-induced nuclear exclusion of FoxO3/4 in GCs was examined in this work. Structural analysis has shown that all FOXO proteins contains a nuclear export sequence domain (NES) and a C-terminal transactivation domain (TAD) as well as one or two of the nuclear localization sequences (NLS) that contains the PKB motifs to be phosphorylated and a forkhead winged helix-turn-helix DNA binding domain [65,66]. Of which, the NLS domain mainly functions to shift FOXO towards the nucleus [67]; whereas phosphorylation of the PKB site in the forkhead domain leads to disruption of the NLS function and cytoplasmic retention of FOXO3 [68]. On the other hand, the NES domain was found to interact with the chromosomal region maintenance protein 1(CRM1), an evolutionarily conserved exportin 1 homolog that was shown to be a nuclear export

receptor for NES-dependent protein transport [69–71]. Moreover, previous studies have proven that the NES is required for the translocation of FOXO3/4 from nucleus to cytoplasm [40,72]. The C-terminus of FOXOs containing a PKB phosphorylation site and a 14-3-3 binding motif, serves as a nuclear localization signal and the phosphorylation of FOXO3/4 leads to their translocation from nucleus to cytoplasm [73–75]. In the present study, the result of double immunofluorescence microscopy further showed that both FoxO3 and FoxO4 were distributed in the GC nucleus and cytoplasm, while the FOXO3/4 were almost failed to be observed in nuclei but accumulated in the cytoplasm of the GCs treated with FSH for 12h. According to our best knowledge, it is the first time that this study has identified the effect of FSH stimulation on nuclear exclusion of FoxO3/4 in the cultured GCs of hen ovarian follicles. However, as the FOXO proteins are constantly exported from the nucleus in a CRM1-dependent manner, CRM1 inhibitors, such as LMB, can be used to demolish the nuclear export [76,77]. Here, it was found that as the levels of FOXO3/4 phosphorylation induced by FSH were increased dramatically in the GCs, simultaneously the nuclear exclusion of FOXO3 and FOXO4 occurred. But this stimulatory effect was dramatically abrogated by LMB. Obviously, this data in chicken ovary has provided another new evidence for the phosphorylation of FOXO3/4 to bring about the translocation the FOXO3/4 proteins from nucleus to cytoplasm because of the phosphorylation directly leading the retention of FOXO3/4 factors, and meanwhile the cellular relocation of the FOXO3/4 towards to cytoplasm being abolished by LMB as the aforementioned [68,73–75]. And this subcellular localization of FOXO3/4 is important for their activities and functions [65,75].

Additionally, this study demonstrated that the expression levels of the cell proliferation-related factors, including *MYC*, *PCNA* and *BCL2* transcripts were dramatically enhanced in the GCs by the treatment of FSH, whereas the expression level of the *CASP3* mRNA was significantly reduced, and this concomitantly resulted in a remarkable decrement in apoptosis of the GCs. The current data corresponds to the view that FOXO factors function as a tumor suppressor in human and mammals, which has been shown to regulate expression of genes implicated in cell proliferation, cell cycle arrest and apoptosis [25,41,43], and phosphorylation or nuclear exclusion of the FOXO is critical to promote cell proliferation and differentiation, but to inhibit apoptosis [39,64,78]. Intriguingly, it has earlier been reported that FOXO3 is a substrate of caspase-3-like proteases [79]; but the expressions of the *CASP3* and *BCL2* genes in this work were found to be modulated by FOXO3 and FOXO4 proteins, which directly influence the GC proliferation and apoptosis. These findings give us a notion that there might be a potential feedback-regulatory loop involving interactions between the FOXO3/4 factors and the caspase-3-like proteases, by which the normal homeostasis, cellular proliferation and differentiation of the prehierarchical follicles were maintained during chicken ovary development. A further study of elucidating the feedback mechanism is going on.

5. Conclusions

In summary, the current study initially demonstrated that expression levels of the phosphorylated FOXO3 and FOXO4 were significantly increased in the cultured GCs from the ovarian follicles challenged by FSH in chicken, whereas the expression levels of non-phosphorylated FOXO3/4 markedly decreased. Simultaneously, the FSH-induced nuclear translocation of FOXO3/4 in GCs was found, suggesting the phosphorylation of FOXO3/4 leads to their nuclear exclusion. Furthermore, the nuclear exclusion of FOXO3 and FOXO4 dramatically enhanced the expression of the *MYC*, *PCNA*, *BCL2*, *STAR* and *CYP11A1* transcript levels but significantly reduced the expression of the *CASP3* mRNA level in a similar manner, which concomitantly results in a memorable augment in cell proliferation but a remarkable decrement in apoptosis of the GCs. These findings provided novel evidence that the FSH-induced nuclear exclusion of FOXO3/4 promotes GC proliferation while attenuates the apoptosis of GCs in hen ovarian follicles.

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