

# Fibromodulin is involved in autophagy and apoptosis of granulosa cells affecting the follicular atresia in chicken

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**ABSTRACT** Follicular atresia is an important cause of reproductive decline in egg-laying hens. Therefore, a better understanding of the regulation mechanism of follicle atresia in poultry is an important measure to maintain persistent high egg performance. However, how the role of the regulatory relationship between autophagy and apoptosis in the intrafollicular environment affects the follicular atresia of chickens is remain unclear. The objective of this study was to explore the regulatory molecular mechanisms in regard to follicular atresia. 20 white leghorn layers (32-wk-old) were equally divided into 2 groups. The control group was fed freely, and the experimental group induced follicular atretic by fasting for 5 d. The results showed that the expression of prolactin (PRL) levels was significantly higher in the fasted hens, while the levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) were lower. Most

importantly, RNA sequencing, qPCR, and Western blotting detected significantly elevated levels of autophagy and apoptosis markers in atresia follicles. Interestingly, we found that fibromodulin (FMOD) levels was significantly lower in follicles from fasted hens and that this molecule had an important regulatory role in autophagy. FMOD silencing significantly promoted autophagy and apoptosis in granulosa cells, resulting in hormonal imbalance. FMOD was found to regulate autophagy via the transforming growth factor beta (TGF- $\beta$ ) signaling pathway. Our results suggest that the increase in autophagy and the imbalance in internal homeostasis cause granulosa cell apoptosis, leading to follicular atresia in the chicken ovary. This finding could provide further insight into broodiness in chicken and provide avenues for further improvements in poultry production.

**Key words:** autophagy, apoptosis, follicle atresia, granulosa cell, chicken

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## INTRODUCTION

One physiological phenomenon in fowl reproduction is ovarian follicle atresia, during which a large number of follicles degenerate and are reabsorbed by the body (Tilly et al., 1991). Follicular atresia involves the degeneration and clearance of follicles before ovulation (Sakamaki et al., 1997). There are significant changes in the levels of reproductive endocrine hormones, such as

prolactin (PRL), luteinizing hormone (LH), progesterone and estradiol, along with changes in the expression of their related genes in the process of follicular atresia (Yao et al., 1998). During follicular atresia, follicles begin to shrink and deform; their shape changes from round to irregular, their color changes from bright to dull gray, and obvious bleeding spots become visible (Moley and Schreiber, 1995). The occurrence of follicular atresia in poultry is closely related to the regulation among granulosa cells, membrane cells, oocytes, or secondary oocytes (Kitamura et al., 2002). Accumulating evidence has shown that follicular atresia is mainly caused by the apoptosis of follicular cells, primarily granulosa cells, which are necessary for follicular development (Hsueh et al., 1994). However, the apoptosis of follicular granulosa cell is complex and involves multiple factors, such as hormones (Erbaş et al., 2014), cytokines

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and oxidative stress (Stanley et al., 2013), all of which play important roles in this process. Interestingly, in recent years, there has been increasing evidence showing that autophagy plays a unique phenotypic role in follicular development and atresia.

Autophagy is an integral degradation process in which cytoplasmic proteins and damaged organelles are phagocytosed by double-membrane vesicles that then fuse with lysosomes to form autophagy lysosomes. These proteins and organelles are then degraded by acid hydrolases and proteases in lysosomes in order to achieve cell homeostasis and organelle renewal (Mizushima and Komatsu, 2011). Autophagy is activated under conditions of external stress, such as nutritional deficiency and hypoxia, and it promotes cell survival by degrading cellular components to release energy and eliminating defective or damaged organelles and proteins (Desideri et al., 2014). Autophagy is also involved in other physiological pathways such as apoptosis. Like autophagy, apoptosis plays a fundamental role in cell development. In fact, autophagy and apoptosis involve common proteins, and autophagy plays an important role in the regulation of apoptotic cell death (Maiuri et al., 2007). Apoptosis is the root cause of follicular atresia in birds and mammals, and granulosa cell apoptosis triggers follicular atresia (Zhao et al., 2014). In this study, we investigated the roles of autophagy and apoptosis in the process of follicular atresia, and identified the molecular regulatory mechanisms of autophagy regulatory molecule FMOD in the process of follicular atresia in chicken.

## MATERIALS AND METHODS

### Animals

All animal testing procedures have been approved by the Institutional Animal Care and Utilization Committee of Sichuan Agricultural University, and the approval number was 2018102021. The chickens used in this experiment were 20 white leghorn layer (32 wk of age) bred in the breeding farm of Sichuan Agricultural University. Individually caged chickens housed in a temperature-controlled room (maintained at 20°C–22°C) on a 14L:10D photo-period throughout the experiment. The hens were randomly divided into 2 groups: control group ( $n = 10$ ), which were given free feed intake add, experimental group ( $n = 10$ ), which was subjected to pause in laying by complete feed withdrawal for 5 d as previously described (Leśniak-Walentyn and Hrabia, 2016). Egg production rate and ovarian weight were recorded. Under pentobarbital sodium anesthesia, ovarian tissue was removed from the abdominal cavity, stripped in a disinfected petri dish. The ovary was washed twice in PBS to clean the connective tissue and blood around the follicle. The follicle was then cut with a surgical blade and the yolk was gently extruded to separate the granular layer and membrane layer of the follicle. The follicles were collected according to the diameter of the follicle, white follicles (1–5 mm, have not entered the

hierarchy), small yellow follicles (5–10 mm, have not entered the hierarchy), and large yellow follicles (>10 mm, have entered the hierarchy). From control groups, we collected the theca and granulosa layers from the preovulatory follicles. In the fasted hens, the layers cannot be separated due to follicle atresia, the walls of these follicles were set as theca. The collected samples were frozen in liquid nitrogen and then stored in a -80°C freezer for further analysis.

### Chicken Granulosa Cells Isolation and Culture

The F1, F2, and F3 ovarian follicles of laying hens were isolated and placed in PBS (Sigma-Aldrich, St. Louis, MO). The chicken granulosa cells were separated from the membrane layer as previous described (Gilbert et al., 1977). After collecting granulosa layer, with 0.1% of collagenase II (Sigma) dispersive granulosa cells, at 37°C water bath for 10 min. Then the cells were collected by centrifugation at  $1500 \times g$  and added to the fresh medium. Subsequently, Cell suspension was seeded into 24-well culture plates at a density of  $1 \times 10^6$  cells/well. The cells were grown on DMEM (Sigma), 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY) and 0.5% streptomycin (Sigma) at 37 °C under 5% CO<sub>2</sub>. After 72 h of cell culture, RNA and proteins were collected for further analysis.

### FMOD Knockdown

To silence fibromodulin (FMOD) in chicken granulosa cells, cell cultures were grown in 24-well plates to a density of 90%. The cells were then transfected with FMOD and Ctrl siRNA, respectively. The transfection reagent is Lipofectamine3000 (Invitrogen) and transfection is performed according to the manufacturer's instructions. The interference effect of FMOD was confirmed by the analysis of FMOD mRNA and protein by qPCR and western blot respectively.

### RNA Extraction and Real-Time Quantitative RT-PCR

Total RNA was extracted from follicular tissues and granulosa cells using TRIzol reagent (TakaRa, Tokyo, Japan), the genomic DNA was then treated with RNase-free DNase to remove the contamination. Reverse-transcribed approximately 2 ug total RNA using the Takata reverse transcription kit according to the manufacturer's instructions. The cDNA was stored in a freezer at -20°C for qPCR analysis of mRNA expression. Real-time quantitative PCR examined FMOD, ATG5, ATG7, Beclin1, caspase-3, caspase-8, and caspase-9 mRNA expression levels in a CFX96-TouchTM Real-Time System (Bio-Rad, Hercules, CA) according to the previous described (Han et al., 2019). The primers used in this experiment are shown in Table 1.

**Table 1.** Gene-special primers for RT-PCR.

Genes <sup>1</sup>	Orientation	Primer sequences (5'-3')	Product size	Accession number
FMOD	Forward	ATGGGCAGCACATCTCGATT	118	NM_204967.1
	Reverse	TCTTCGGCTTTGCAGGTCAT		
Caspase 9	Forward	TATGGTGGAGGACATGCAGA	99	XM_424580.5
	Reverse	AATATTGGGAAGGCCTGCTT		
Caspase 3	Forward	AAAGATGGACCACGCTCAGG	204	NM_204725
	Reverse	TGAACGAGATGACAGTCCGG		
Caspase 8	Forward	GCTGTATCCTATCCCACG	125	KM_016991
	Reverse	TCATCAGGCACCTCCTTT		
ATG5	Forward	GATGAAATAACTGAAAGGGAAGC	124	NM_001006409.1
	Reverse	TGAAGATCAAAGAGCAAACCAA		
ATG7	Forward	TCAGATTCAAGCACTTCAGA	62	NM_001030592.1
	Reverse	GAGGAGATAACAACCACAGAG		
Beclin1	Forward	CAGACACGCTGCTGGACC	84	NM_001006332.1
	Reverse	TCTCCTTGTCATCCTCGTTCA		
GAPDH	Forward	TCCTCCACCTTTGATGCG	99	NM_204305.1
	Reverse	GTGCCTGGCTCACTCCTT		

### Western Blot Analysis

Proteins from granulosa cells and follicles were collected by protein extraction kit and protein quantified by BCA (BestBio, Shanghai, China) method. First, the proteins were bathed in a metal bath at 95°C for 5 min. The lysate containing 20  $\mu$ g total protein was separated by SDS-PAGE (Beyotime, Shanghai, China). The lysate was washed with PBS and transferred to PVDF membrane (Beyotime). After sealing with a blocking solution, the membrane was incubated overnight with the primary antibody at 4°C. The membrane was then washed in TBS for 3 times for 15 min each and incubated with antirabbit secondary antibody or antimouse secondary antibody at room temperature for 2 h. After washing with TBST, the protein was visualized by enhanced chemiluminescence (Beyotime). Density analysis of the bands relative to GAPDH protein was performed using ImageJ software. The following primary antibodies were used: rabbit anti-LC3B (cat. no. 3868T, Cell signaling Technology), mouse anti-Beclin1 (cat. no. 200675, ZenBio), rabbit anti-FMOD (cat. no. bs-12362R, Bioss), mouse anticaspase3 (cat. no. bsm-33284M, Bioss), rabbit anticaspase8 (cat. no. Asp391, CST), and mouse anti-GAPDH (cat. no. bsm-33033M, Bioss).

### Immunofluorescence Assay

After the tissue was collected, it was washed twice with PBS and then fixed with 4% paraformaldehyde for 30 min. To examine the LC3B expression levels in follicles from egg-laying and fasted hens, immunofluorescence staining was performed on the tissue sections according to previous methods (Pelosi et al., 2013). To perform immunofluorescence on the tissues, the dewaxed sections were first microwaved in Tris-EDTA buffer. The tissue sections fixed with 4% PFA were washed with PBS and permeated with 0.5% Triton X-100 for 10 min. The tissue sections were blocked with goat serum (Gibco, Grand Island, NY) at room temperature for 1 h and then incubated with LC3B antibody at 4°C overnight. The TBS was washed twice and incubated

with the secondary antibody for 1 h at room temperature. The nuclei were shown with DAPI staining. Then the fluorescence intensity was measured under a fluorescence microscope. Mean = IntDen/Area. Mean: Mean gray value; IntDen: Integrated Density;

### Plasma Collection and Hormone Analysis

Approximately 2 mL volume of blood was collected from each chicken through venipuncture. After centrifugation at 2000 rpm for 15 min, the plasma was separated from the supernatant of the blood and stored at -20°C. Plasma concentrations of prolactin (PRL), luteinizing hormone (LH) and follicle stimulating hormone (FSH) were measured using a chicken specific ELISA kit (Jian-Cheng Inc., Nanjing, China). All analyses were performed according to kit protocol.

### Flow Cytometry Analysis

To induce or inhibit autophagy in chicken granulosa cells, cells were treated with autophagy inducer rapamycin (RAP) (4  $\mu$ M, Sigma) for 6 h and inhibitor 3-Methyladenine (3-MA) (5 mM) for 6 h. Chicken granulosa cells were harvested by centrifugation (15 min at 1500 rpm at 4 °C), then washed with PBS. The 100  $\mu$ L solution was then transferred to a 5 mL culture tube, added with 5  $\mu$ L Annexin V-FITC (Cat.no. 51-65874X) and 5  $\mu$ L PI (Cat.no. 51-66211E), respectively. The culture tube containing the cells was then vortexed gently and incubated in darkness at room temperature for 15 min. Then, we added 400  $\mu$ L 1 X binding buffer to each tube, and then the apoptosis rate was analyzed by flow cytometry (BD FACSCalibur).

### Transmission Electron Microscopy

Cells were detached from the dish using a manual scraper, washed 3 times with PBS. Then, the cells were suspended and fixed in 2% glutaraldehyde with 1% tannic acid in 0.1 M sodium cacodylate overnight at 4°C. The cells were washed 3 times in sodium carbonate

buffer and then incubated in the same buffer with 2% osmium tetroxide at room temperature for 1 h. The cells were then washed 3 times in distilled water and exposed to 1% uranyl acetate water for 15 min at room temperature. The cells were then washed twice, spun into 3% agarose at 45°C, and cooled to form blocks. The agarose blocks were graded dehydrated with acetone and embedded in Spurr's low viscosity medium. After overnight polymerization at 65°C, sections at 80 nm were cut on a Reichert-Jung Ultracut E micromicroslicer (Leica Microsystems, Milan, Italy) and collected on a copper wire. The sections were observed on a HITACHI HT7800/HT7700 TEM (HITACHI, Tokyo, Japan) and micrographs were recorded on a Kodak 4489 sheet film.

### **RNA-seq and Data Analysis**

Total RNA were prepared using TRIzol. Then, the RNA samples were submitted to Beijing Novogene technology Co., Ltd for library construction, sequencing and bioinformatics analysis in strict accordance with the company's standard operating procedures, which are available online (<https://www.novogene.com/>).

### **Statistical Analysis**

The values were expressed as mean  $\pm$  standard error of mean (s.e.m). Student's paired *t*-test was used to determine the significance of the difference between 2 groups. Nonparametric one-way ANOVA with SPSS (SPSS Inc., version 13.0.1 for windows, Chicago, IL) was used to compare the statistical data between the groups, and *P* < 0.05 was considered statistically significant.

## **RESULTS**

### **Morphological Follicular Changes and Change in Hormone Levels in Egg-Laying and Fasting Hens**

We found hens completely stop laying after 5 d of fasting (Figure 1A). Compared with the control group, the fasting hens decreased 56% of their ovarian weight (Figure 1B). The yellow hierarchical follicles in the ovaries of fasting hens are atretic and often hemorrhagic (Figure 1C). Plasma concentration tests showed that the blood plasma of PRL levels were significantly lower in egg-laying hens than fasted hens, while LH and FSH levels were higher (Figure 1D).

### **Expression of Autophagy and Apoptosis Markers Between Normal Follicles and Atresia Follicles**

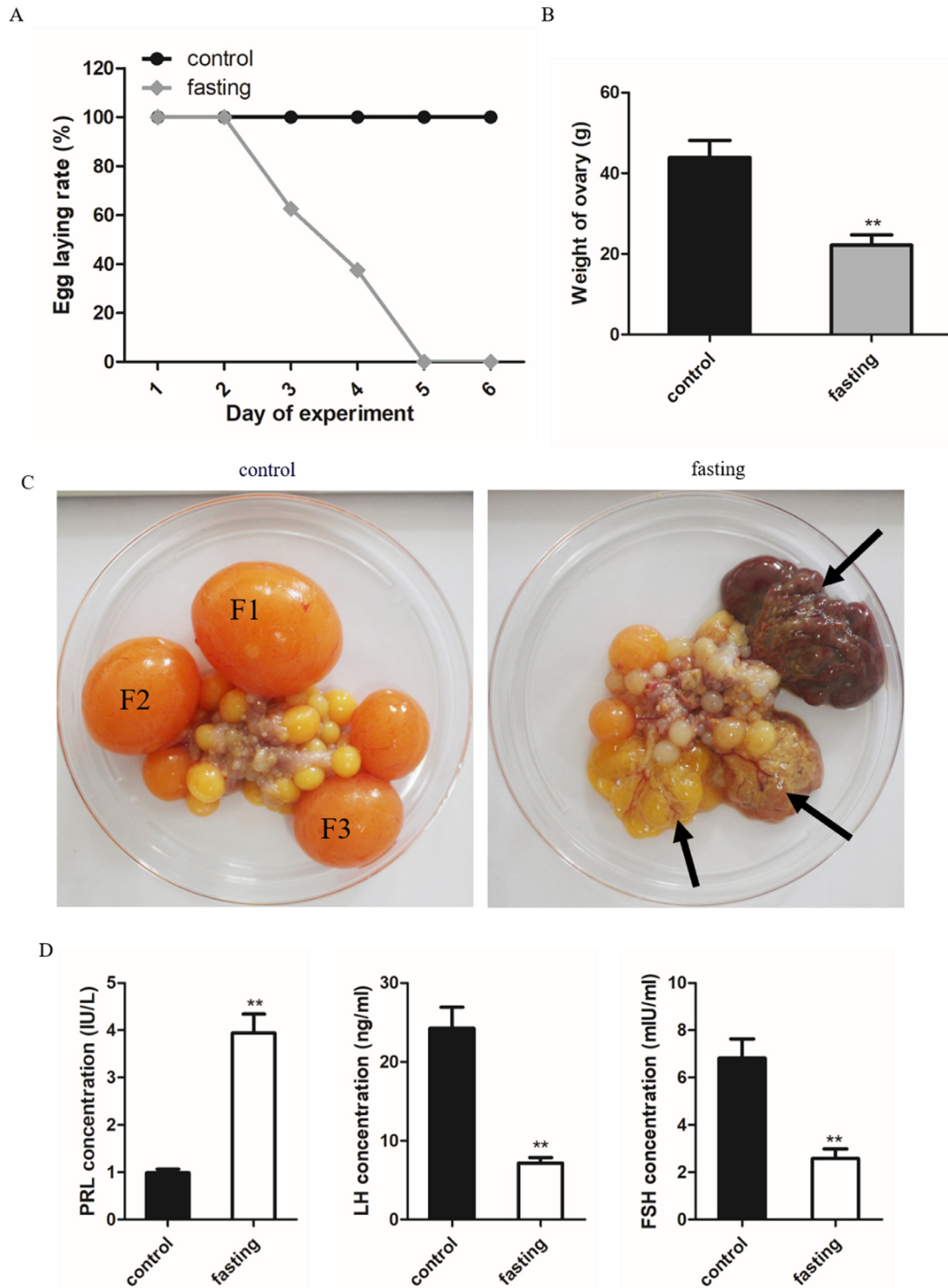
To further explore the molecular regulatory mechanisms underlying follicular atresia, we performed RNA sequencing using follicles from fasted and egg-laying hens. Among the 22,315 expressed transcripts, 627 with Ref-Seq annotations showed significant differences in

expression between follicles from egg-laying and fasted hens. Among these different genes, a series of genes involved in autophagy and apoptosis were found, which may play an important role in follicular atresia (Figure 2A). We verified the transcriptome results using qPCR and found that the autophagy-related genes autophagy related 5 (Atg5), autophagy related 12 (Atg12) and Beclin1 and the apoptosis-related genes caspase-3, caspase-8 and caspase-9 showed significantly higher expression in follicles from fasted hens (Figure 2B). KEGG analysis showed that the identified differentially expressed genes showed enrichment in autophagy and apoptosis signaling pathways, suggesting that autophagy and apoptosis play an important regulatory role in the development and atresia of hen follicles (Figure 2C). Immunofluorescence showed that the expression of LC3-II in fasted hen follicles were significantly higher than that in control group (Figure 2D and E). Western blot also showed that the protein expression of LC3B and Beclin1 was higher in follicles from fasted hens (Figure 2F). Using electron microscopy, we found that follicles from fasted hens had a significantly higher number of apoptotic bodies than follicles from egg-laying hens (Figure 2G). We also found that the protein levels of caspase-8 and caspase-9 were higher in follicles from fasted hens (Figure 2H). Together, these results indicated follicles from fasted hens exhibited higher levels of autophagy and apoptosis markers than follicles from egg-laying hens.

### **Effects of FMOD on Autophagy and Apoptosis in Chicken Granulosa Cells**

A comparative analysis of follicles from egg-laying and fasted hens showed a 20-fold difference in FMOD expression, and the differences in expression was confirmed via qPCR analysis (Figure 3A). Subsequently, we cultured chicken primary granulosa cells in vitro to study the effect of FMOD silencing on granulosa cells (Figure 3B and C). qPCR assays showed that after FMOD silencing, prolactin receptor (PRLR) levels significantly increased and follicle stimulating hormone receptor (FSHR), and luteinizing hormone receptor (LHR) levels significantly decreased (Figure 3D). We found that the autophagy-related genes Beclin-1, Atg5, and Atg7 showed increased expression after FMOD silencing (Figure 3E). Western blot analysis further demonstrated that FMOD silencing significantly increased LC3-II and beclin1 levels (Figure 3F). The apoptosis-related genes caspase3, caspase-8 and caspase-9 were significantly upregulated in chicken granulosa cells transfected with FMOD siRNA (Figure 3G). The apoptosis of granulosa cells was significantly increased after FMOD silencing, as confirmed by flow cytometry (Figure 3H). Electron microscopy showed that the number of apoptotic bodies also increased significantly after FMOD silencing (Figure 3I). Finally, western blot demonstrated that caspase-8 and caspase-9 protein levels showed significantly increased in FMOD silenced cells (Figure 3J).



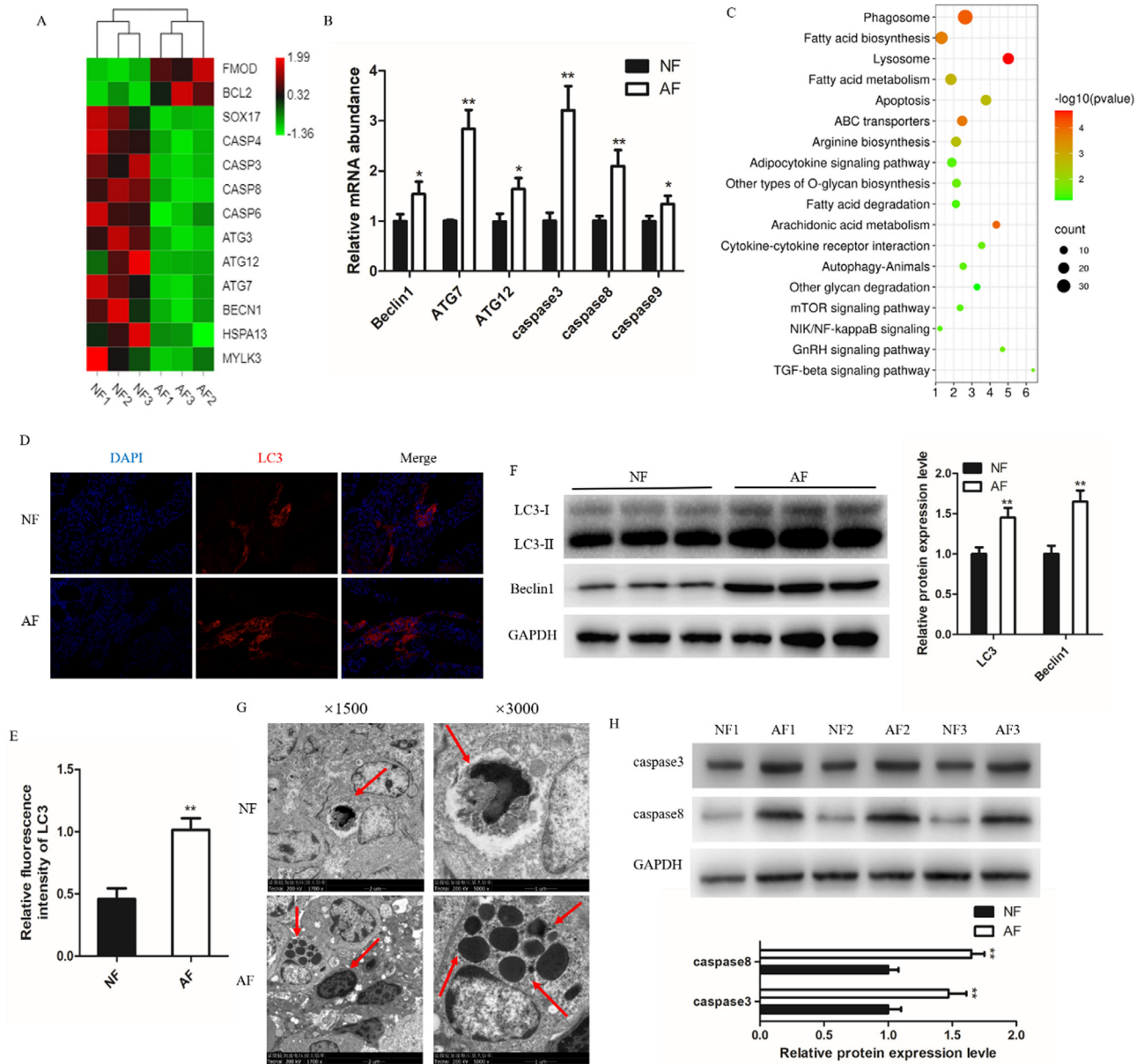


**Figure 1.** Morphological characteristics of ovary and plasma PRL, LH and FSH hormone expression levels in follicles from egg-laying and fasted hens. (A) The rate of egg production during a pause in egg production caused by fasting. (B) The weight of the ovary during the egg production pause induced by fasting. (C) Morphological characteristics of ovary during a pause in egg production caused by fasting. Abbreviations: F3<F2<F1), the 3 largest yellow preovulatory follicles. The arrow points to yellow follicle were atretic. (D) Detection of PRL, LH and FSH hormone levels in plasma during a pause in egg production caused by fasting. Data represent mean  $\pm$  S.E. n = 3 hens in each group. \* $P < 0.05$ , \*\* $P < 0.01$ .

### **FMOD Protects Cells Against Apoptosis by Regulating Autophagy**

In order to further investigate the effects of FMOD on autophagy and apoptosis of chicken granulosa cells, we treated FMOD-silenced cells with the autophagy inducer rapamycin (RAP) and inhibitor 3-

methyladenine (3-MA). We found that FMOD-silenced cells showed significantly higher levels of apoptosis after RAP treatment, while apoptosis was alleviated after 3-MA treatment (Figure 4A and B). Western blot analysis showed that RAP treatment promoted the expression of caspase3 protein, while 3-MA treatment inhibited its expression in FMOD-silenced cells (Figure 4C). These



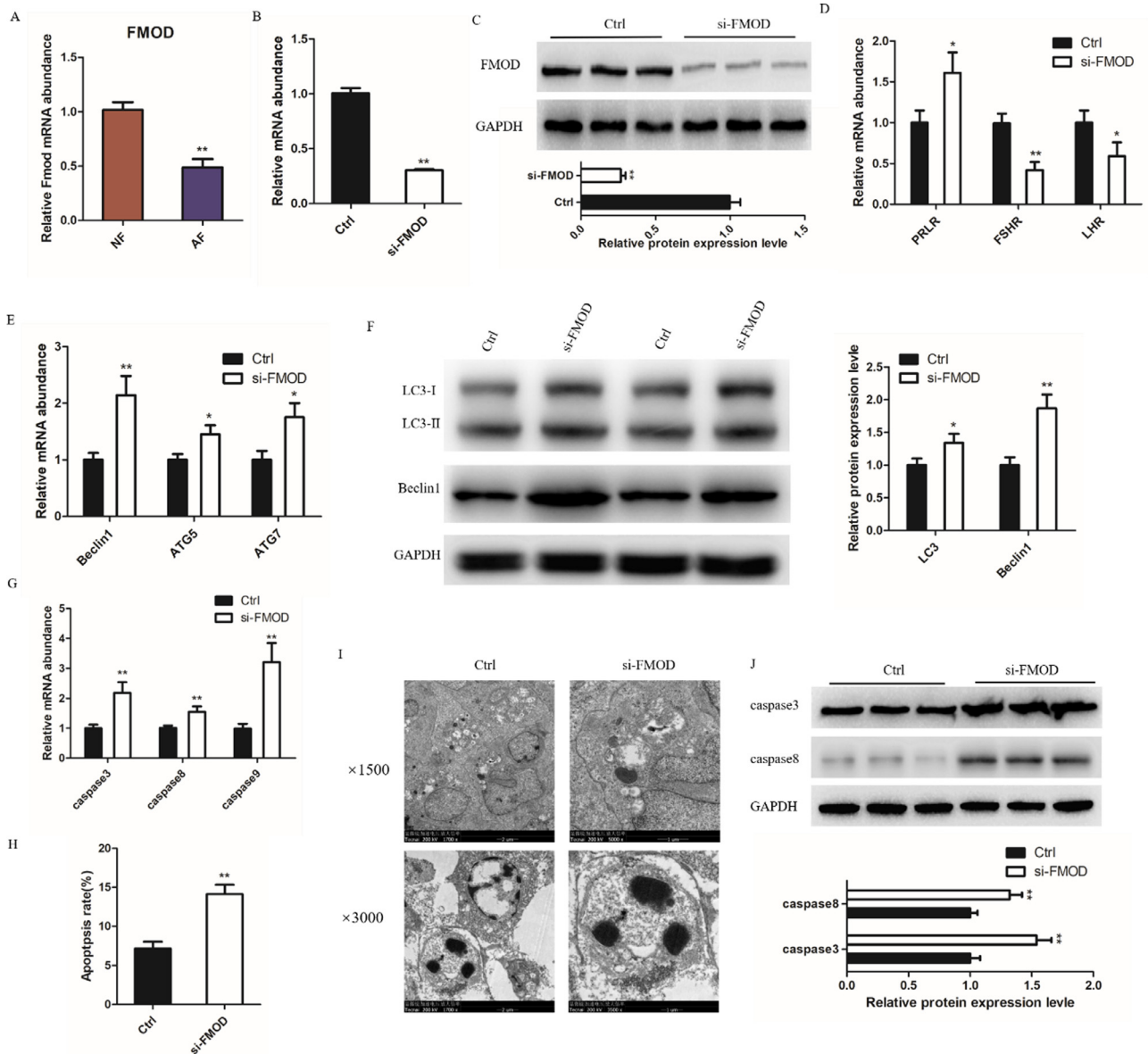
**Figure 2.** Expression levels of autophagy and apoptosis in normal follicles and atresia follicles. (A) Hierarchical clustering and heatmap of significant difference genes between normal follicles and atresia follicles. (B) Relative mRNA expression levels of Beclin1, ATG7, ATG12, caspase-3, caspase-8 and caspase-9 in follicles from egg-laying and fasted hens. (C) Pathway enrichment analysis of significant differential expression genes between follicles from egg-laying and fasted hens. X-axis is the enrichment factor. (D) Immunofluorescence analysis of LC3 expression levels in normal follicles and atresia follicles. (E) The right diagram shows the relative fluorescent intensity of LC3 in normal follicles and atresia follicles. (F) Western blot analysis the protein expression levels of LC3B and Beclin1 in normal follicles and atresia follicles. The histogram on the right shows the gray value analysis of protein. (G) Apoptotic bodies in normal follicles and atresia follicles were observed by electron microscopy. (The red arrows indicate apoptotic bodies). (H) Western blot analysis the protein levels of caspase-3 and caspase-8 in normal follicles and atresia follicles. The histogram below shows the gray value analysis of protein. Data represent mean  $\pm$  S.E.  $n = 3$  hens in each group. \* $P < 0.05$ , \*\* $P < 0.01$ . Abbreviations: AF, atresia follicles; NF, normal follicles.

results indicated that FMOD silencing may induce atresia in granulosa cells by enhancing autophagy in order to promote follicular apoptosis.

### Effects of FMOD on TGF- $\beta$ Signaling Pathway

To investigate the molecular mechanisms via which FMOD regulates follicular atresia, we performed RNA-

sequencing of FMOD-silenced and control granulosa cells, and screened a series of genes involved in autophagy and apoptosis (Figure 5A). KEGG enrichment analysis of the identified genes revealed enrichment in the TGF- $\beta$  signaling pathway, which is known to play an important regulatory role in autophagy (Figure 5B). Using qPCR, we found that FMOD silencing significantly promoted the expression of TGF- $\beta$ 1 and INHBA (Figure 5C). Western blot analysis also revealed increased levels of TGF- $\beta$ 1 protein in FMOD silenced cells (Figure 5D).



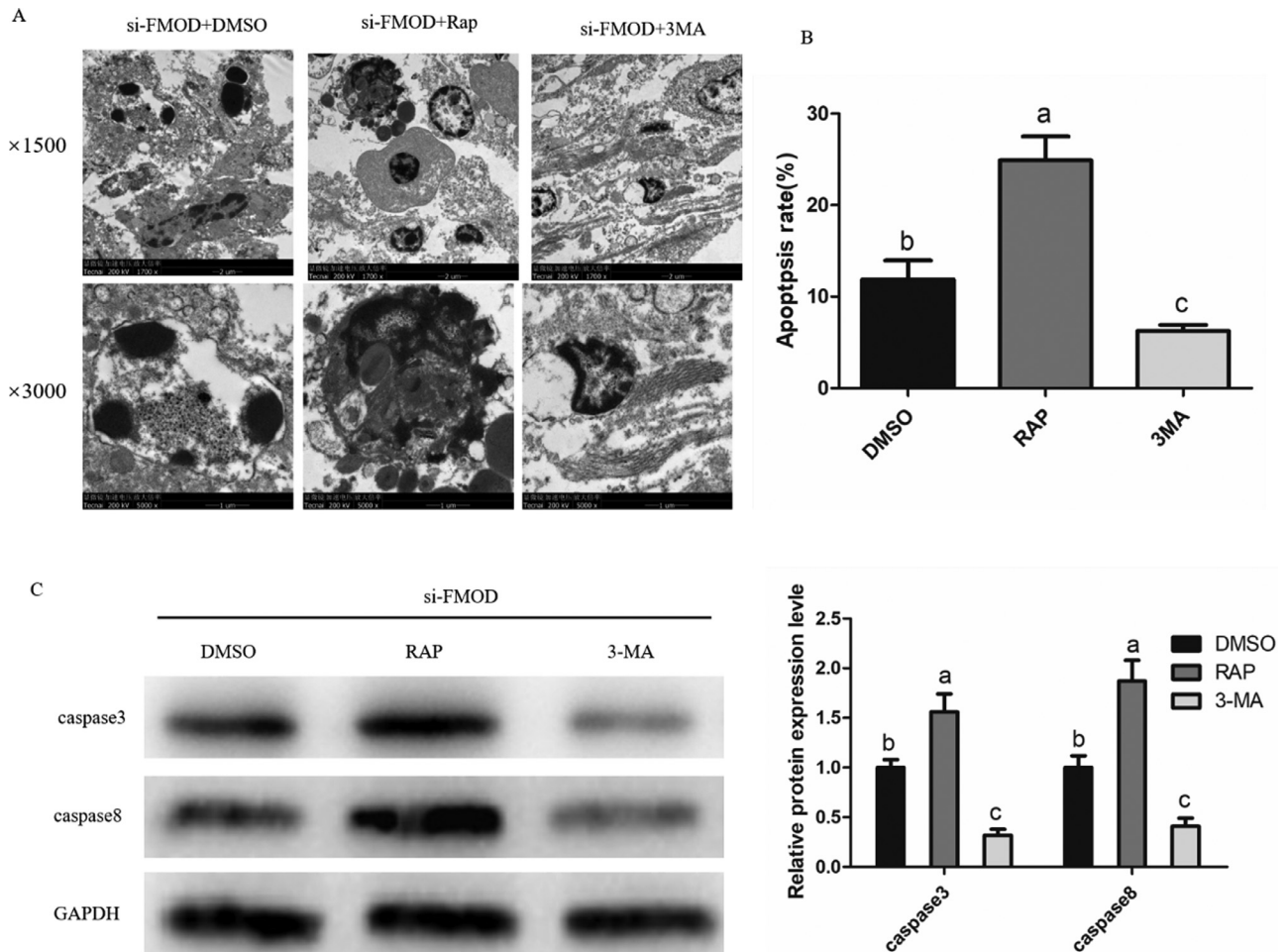
**Figure 3.** Effects of FMOD on autophagy and apoptosis in chicken granulosa cells. (A) Relative FMOD mRNA expression levels in normal follicles and atresia follicles. (B) Relative mRNA expression levels of FMOD in ctrl and FMOD silenced cells. (C) Relative mRNA expression levels of PRLR, FSH- $\beta$  and LH- $\beta$  in ctrl and FMOD silenced cells. The histogram below shows the gray value analysis of protein. (D) Western blot analysis FMOD protein levels in ctrl and FMOD silenced cells. (E) Relative mRNA expression levels of Beclin1, ATG5 and ATG7 in ctrl and FMOD silenced cells. (F) Western blot analysis the expression protein levels of LC3B and Beclin1 in ctrl and FMOD silenced cells. The histogram on the right shows the gray value analysis of protein. (G) Relative mRNA expression levels of caspase-3, caspase-8 and caspase-9 in ctrl and FMOD silenced cells. (H) Apoptosis rates in ctrl and FMOD-silenced cells. (I) The ultrastructure of apoptotic bodies was observed by electron microscopy. (J) Western blot analysis the expression protein levels of caspase3 and caspase8 in ctrl and FMOD silenced cells. The histogram below shows the gray value analysis of protein. Data represent mean  $\pm$  S.E. n = 3 independent cell cultures in each group. \* $P$  < 0.05, \*\* $P$  < 0.01.

## DISCUSSION

In mammalian and avian ovaries, most follicles undergo atresia and die, and only a small percentage mature fully and participate in ovulation (Evans, 2003). Although follicular atresia occurs repeatedly throughout the ovarian cycle, the exact mechanism underlying mass cell death is not fully understood. In this study, we found the expression of PRL levels was significantly higher in the fasted hens, while the levels of LH and FSH were lower. FSH and LH are known to be key hormones in the control of reproduction and stimulate gonadal steroid production or secretion (Dunn et al., 2003). More specifically, LH regulates sex steroid production and

ovulation, while FSH promotes follicular maturation. In addition, there have been reports that the plasma PRL concentration increase with the occurrence of broodiness, and PRL receptor mRNA level is up-regulated during incubation, indicating that PRL is important for the onset of broodiness (Wong et al., 1992; Jiang et al., 2005). As previously reported, fasting results in degeneration of epithelium and involution of tubular glands in the magnum, isthmus and shell gland and follicular atretic in hens (Socha and Hrabia, 2019; Wolak and Hrabia, 2021). We demonstrated the effect of autophagy and apoptosis on follicles from egg-laying and fasted hens. In addition, we found that FMOD confers protection against the apoptosis of chicken granulosa cells via





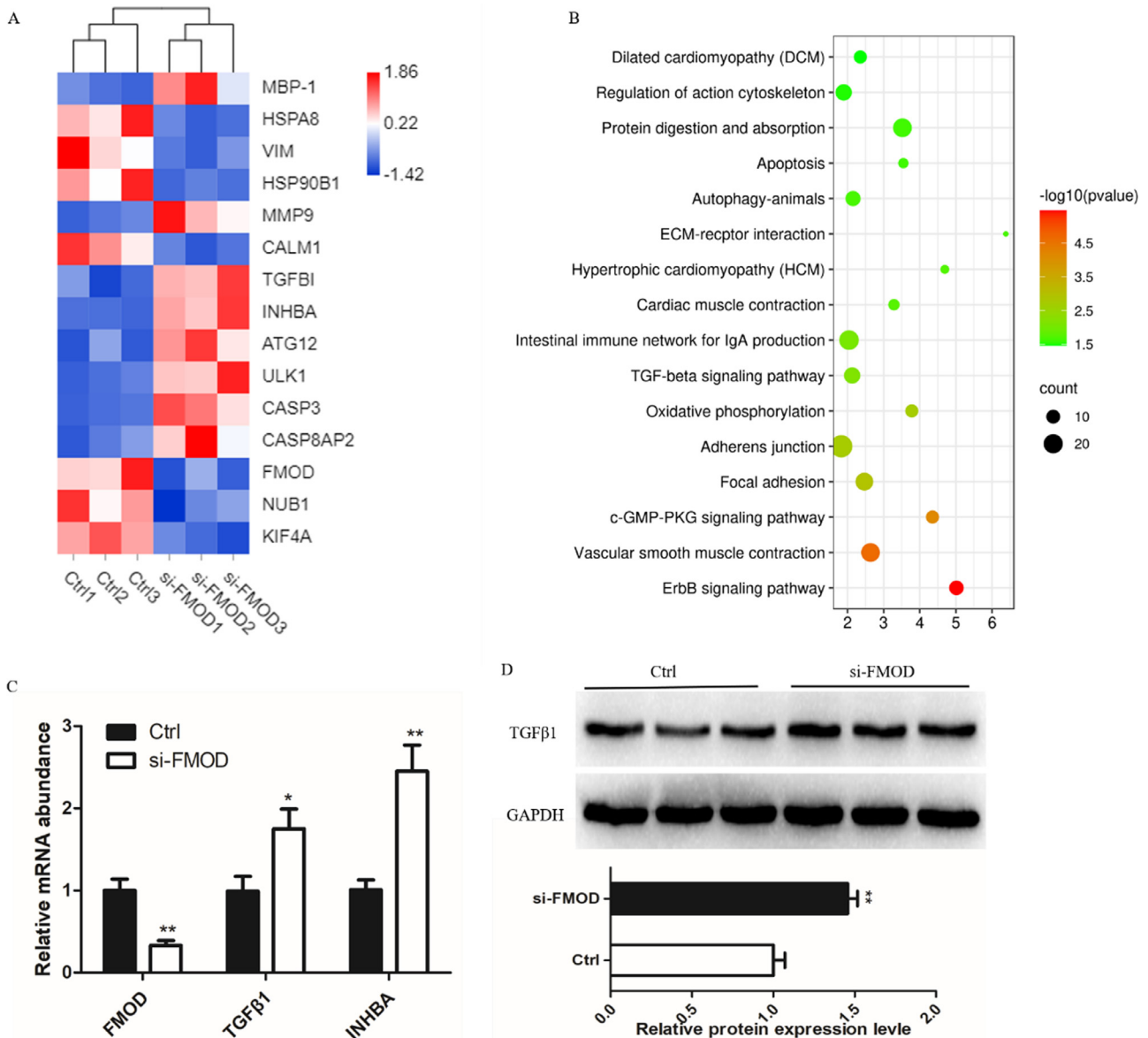
**Figure 4.** FMOD regulates the effect of autophagy on apoptosis. (A) Electron microscopy observed the apoptotic bodies in FMOD-silenced cells after treated with Rap, 3-MA, and DMSO, respectively. (B) Flow cytometry detected apoptosis rates of FMOD silenced cells after treated with Rap, 3-MA, and DMSO. (C) Western blot analysis the expression protein levels of caspase3 and caspase8 in FMOD-silenced cells after treated with Rap, 3MA and DMSO. The histogram on the right shows the gray value analysis of protein. Data represent mean  $\pm$  S.E.  $n = 3$  independent cell cultures in each group. Bars not sharing the same letter labels are different ( $P < 0.05$ ).

autophagy regulation, suggesting that FMOD may be the key mediator of follicular atresia in chickens.

Our results suggested that follicular atresia in chickens might be caused by increased autophagy in follicular granulosa cells. Although the endocrine system, including the hypothalamic-pituitary-ovary system, plays an important role in follicular development and atresia, an increasing number of studies with its involvement with follicular autophagy and atresia (Zhou et al., 2019). In present study, we found that autophagy and apoptosis-related genes were significantly up-regulated in follicles from fasted chickens. These results suggest that autophagy and apoptosis are closely related to follicular atresia in chickens, consistent with findings in mammals. It has been found that autophagosome accumulation induces the apoptosis in rat follicular granulosa cells by lowering Bcl-2 expression and subsequently activating caspase (Choi et al., 2011). In addition, it has been reported that autophagy commonly occurs in granulosa cells and shows a good correlation with apoptosis. Moreover, LC3-II expression was found to show a good correspondence with cleaved caspase-3 expression in granulosa cells isolated from PMSG-treated immature rat ovaries (Choi et al., 2010). In addition, strong cleaved caspase-3

staining and LC3 immunoreactivity were observed in granulosa cells from atretic follicles. It has reported that reactive oxygen species (ROS) levels were significantly higher in broody goose hens than in egg-laying hens, and that increased ROS levels induced granulosa cell apoptosis by activating autophagy (Lou et al., 2017). It has been found that tamoxifen (TMX) treatment has a pro-apoptotic effect in cells of the chicken oviduct wall, and it can significantly increase the expression of caspase and thus induce chicken oviduct regression (Socha and Hrabia, 2018). TMX is known autophagy inducer, and several studies have reported that TMX affects cell apoptosis by inducing autophagy (Park et al., 2012; Graham et al., 2016; Kaverina et al., 2018). Therefore, the TMX-induced promotion of apoptosis in chicken oviduct cells may be caused by autophagy. Some researchers cultured chicken granulosa cells in vitro and treated them with leptin found that leptin plays an important role in regulating the proliferation and apoptosis of granulosa cells (Sirotkin and Grossmann, 2007). It has been found that increases in leptin induced by hypoxic preconditioning promote autophagy and protect mesenchymal stem cells from apoptosis (Wang et al., 2014). Therefore, whether leptin can regulate apoptosis in





**Figure 5.** Effects of FMOD silencing on TGFβ signaling pathway. (A) Hierarchical clustering and heatmap of significant difference genes between FMOD silenced and control cells. (B) KEGG pathway enrichment analysis of significant differential expression genes between FMOD silenced and control cells. X-axis is the enrichment factor. (C) Relative mRNA expression levels of FMOD, TGFβ1 and INHBA in control and FMOD silenced cells. The histogram below shows the gray value analysis of protein. (D) Western blot analysis TGFβ1 protein expression levels in FMOD silenced and control cells. Data represent mean ± S.E. n = 3 independent cell cultures in each group. \* $P < 0.05$ , \*\* $P < 0.01$ .

chicken ovary via autophagy warrants further research. These results suggested that chicken follicular atresia might be caused by the increased autophagy of follicular granulosa cells.

Autophagy is regulated by many factors and plays an important role in homeostasis. Under conditions of stress, such as reactive oxygen species (ROS), melatonin, and hypoxia-inducible factor levels, autophagy is activated (Fernández et al., 2015; Li et al., 2015; Ravanan et al., 2017). Moreover, a variety of coding and noncoding genes have also been found to play an important regulatory role in autophagy. Inhibition of nuclear factor-kappa B (NF-κB) increased autophagy in porcine granulosa cells via the JNK signaling pathway and promoted steroid production (Gao et al., 2016). Melatonin protects mouse granulosa cells from oxidative damage and granulosa cell apoptosis by inhibiting FoxO1-

mediated autophagy (Shen et al., 2018). Using the established PMSG stimulation model to induce follicular development in immature rat ovaries and obtain granulosa cells, and found that autophagy plays an important role in regulation of follicular development and atresia (Choi et al 2010). They also found that Akt-mediated mTOR activation can inhibit granulosa cell autophagy during follicular development and contribute to the regulation of apoptotic cell death (Choi et al., 2014). In our study, we found that FMOD can protect chicken granulosa cells from apoptosis by inhibiting autophagy. The expression of FMOD was significantly decreased in atretic follicles, which could explain the increase in autophagy, and resultant induction of apoptosis and atretic. FMOD belongs to the small mesenchymal proteoglycan family. The central region of the protein contains a leucine-rich repeat containing 4 chains of keratin

sulfate, flanked by a terminal domain containing disulfide bonds (Mormone et al., 2012). At present, research on FMOD has largely focused on muscle tissues and tumors (Lee et al., 2018; Pourhanifeh et al., 2019), and its role on follicular development and atresia has not been studied. FMOD is the main regulator of myostatin and can control the progression of satellite cells through myogenic programming (Lee et al., 2016). It has been shown that FMOD inhibits the activity of NF- $\kappa$ B by delaying the degradation of IKBA via a JNK-dependent pathway and thus regulates fibroblast apoptosis (Lee and Schiemann, 2011). It has been found that FMOD silencing promotes apoptosis in lymphocytic leukemia cells (Hassan et al., 2011). Our study also revealed that FMOD silencing promotes the expression of apoptosis-related genes, leading to increased apoptosis of granulosa cells. Further, we found that FMOD silencing promotes the autophagy flux, and that the inhibition of autophagy alleviates the susceptibility of granulosa cells to apoptosis. Therefore, our results indicate that the apoptosis of granulosa cells induced by FMOD silencing maybe autophagy dependent. Our study revealed that FMOD knockdown activates the TGF- $\beta$  signaling pathway. Research has been reported that TGF- $\beta$  plays an important role in the regulation of autophagy (Alizadeh et al., 2018). TGF- $\beta$ 1-induced autophagy is required for the fibrogenesis response in human atrial myofibroblasts (Ghavami et al., 2015). It has been demonstrated that TGF $\beta$ 1-mediated autophagy plays an important role in the pathology of liver fibrosis (Fu et al., 2014). Our results also proved that the TGF- $\beta$  pathway was activated after FMOD silencing and leads to autophagy, which may be an important mechanism via which FMOD regulates the autophagy of granulosa cells and thus regulates follicular atresia.

## CONCLUSION

These findings demonstrated that the autophagy and apoptosis patterns in normal follicles and atresia follicles, and revealed that increased autophagy may contribute to chicken follicular atretic. In addition, we found that autophagy induced by FMOD silencing in follicles from fasted hens may be cause follicular atresia. Taken together, our results provide a theoretical basis for the relationship of autophagy with atresia, and provide a potential target for the treatment of anovulatory disorders.

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Data Availability: The datasets generated and analyzed are available from the corresponding author on reasonable request. The data used in this study have been deposited in the National Center for Biotechnology

Information Sequence Red Archive (SRA) under the accession number PRJNA721929.

## DISCLOSURES

The authors declared that they have no conflicts of interest to this work.

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