MiR-34a-5p promotes autophagy and apoptosis of ovarian granulosa cells via the Hippo-YAP signaling pathway by targeting LEF1 in chicken

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ABSTRACT Follicular atresia is a natural physiological phenomenon in poultry reproduction. It is well known that follicular atresia is caused by both autophagy and apoptosis of granulosa cells. In current experiment, we evaluated the function of miR-34a-5p on autophagy and apoptosis in chicken follicular atresia. First, the follicular atresia model of chicken was successfully constructed by subcutaneous injection of tamoxifen (**TMX**), and found the expression of miR-34a-5p in the atresia follicles obviously increased. Then, we confirmed that miR-34a-5p accelerates autophagy and apoptosis of chicken granulose cells in vitro, and miR-34a-5p could induce apoptosis by mediating autophagy. Mechanistically, lymphoid enhancer binding factor 1 (**LEF1**) was deemed as a target gene for miR-34a-5p. On the contrary, LEF1 overexpression attenuated the autophagy and apoptosis of chicken granular cells. In addition, it was confirmed that the miR-34a-5p/LEF1 axis plays a regulatory role in chicken granulosa cells by mediating the Hippo-YAP signaling pathway. Taken together, this study demonstrated that miR-34a-5p contributes to autophagy and apoptosis of chicken follicular granulosa cells by targeting LEF1 to mediate the Hippo-YAP signaling pathway.

Key words: miR-34a-5p, LEF1, autophagy, apoptosis, Hippo-YAP pathway

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INTRODUCTION

Follicular atresia is a process of follicle degeneration that is regulated by a variety of mechanisms, and it is a normal physiological phenomenon in the animal breeding process. In poultry, follicular atresia occurred at any stage of follicle development, and the number of atresia follicles directly affected the egg laying rate. Apoptosis of follicular granulosa cells is considered as the fundamental cause of follicular atresia (Hughes and Gorospe, 1991). However, recent studies have revealed that follicular atresia is not caused by only apoptosis of granulosa cells, but also through the process from autophagy to apoptosis of granulosa cells. Thus, autophagy could mediate apoptosis to give rise to follicular atresia (Choi et al., 2010; 2011). Kang et al. (2018) found that autophagy and apoptosis proteins are co-activated during follicular atresia, and the interaction between autophagy and apoptosis is the key in determining the fate of fish ovarian cells (Kang et al., 2018). It has been reported that lack of nutrition could lead to autophagy at the stages of germarium and mid-oogenesis in drosophila ovaries, which further results in the death of germ cells and a distinct lower in the amount of oviposition (Hou et al., 2008). Other studies have shown that autophagy was activated and ultimately promoted granulosa cell apoptosis during follicular atresia in hens (Shen et al., 2017). Therefore, follicular atresia is a complex mechanism, which is closely related to autophagy and apoptosis. Hence, studied on follicular atresia in poultry is of great significance in the egg laying performance of hens.

MiRNAs are classes of RNAs with the length of 18 to 24 nucleotides and they perform functions mainly by controlling post-transcriptional genes expression (Vishnoi and Rani, 2017). In recent years, miRNAs are widely reported to participate in a large number of cellular processes, such as follicular development and atresia

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by targeting autophagy-related genes and signaling pathways (Worku et al., 2017). For example, microRNA let-7g was reported in mice to cause autophagy of granulosa cells via mediating insulin-like growth factor 1 receptor (**IGF-1**), and eventually lead to apoptosis (Zhou et al., 2016). MiR-34 is an important cancer suppressor that has attracted a lot of attention. It is reported that miR-34 induced apoptosis by directly targeting the tumor suppressor gene p53 (Chang et al., 2007; Cole et al., 2008). Furthermore, miR-34 contributed to cell cycle arrest and senescence by regulating MYCN proto-oncogene (**MYCN**) (Wei et al., 2008; Silber et al., 2012). Moreover, miR-34 reduced a tumor burden and prolonged survival in a mouse model of hepatocellular carcinoma (Bader, 2012). Combined with the above report, miR-34 is essential for the occurrence of many biological processes. However, the effect of miR-34 is still unclear on autophagy and apoptosis of chicken follicular atresia. Therefore, in this study, we explore that the function of miR-34a-5p in chicken follicular development.

Lymphoid enhancer binding factor 1 (**LEF1**) is a component of the LEF/T-cell factor (**TCF**) family and an important factor of the canonical wingless-type (**Wnt**) signaling pathway. It was reported that LEF1 took part in the procession of cell cycle and proliferation by regulating variety of related-genes (Zhan et al., 2017). LEF1 also played a momentous role in hematopoiesis function (Reya et al., 2000; Petropoulos et al., 2008). In addition, LEF1 regulated the proliferation, migration, invasion of glioblastoma cell, and renewed cancer stem-like cell (Gao et al., 2014). LEF1 is closely related to the Hippo signaling pathway and are determined as key targets for molecular docking (Zhang et al., 2019). The Hippo pathway is a tumor suppressor pathway and controls multifarious physiological processes, including proliferation, apoptosis, and cell survival (Jia et al., 2003). The Hippo pathway was identified as an important regulator of autophagy (Pavel et al., 2021). However, it is not clear whether LEF1 is involve in autophagy and apoptosis of chicken follicular granulosa cells by regulating the Hippo signaling pathway.

In this present study, we successfully induced follicular atresia model by tamoxifen (**TMX**), and revealed that miR-34a-5p was highly expressed in the atretic follicles. Moreover, the molecular regulatory mechanism and the potential targets of miR-34a-5p were detected in the chicken follicular granulosa cells, and all the results showed that miR-34a-5p involves in follicular atresia by targeting LEF1 to modulate the Hippo-YAP signaling pathway in chicken.

MATERIALS AND METHODS

Animals and Sample Preparation

All experimental animals in this study were approved by the Animal Ethics Committee of Sichuan Agricultural University in Yaan, and the permit number is 2020100106.

Thirty-two-wk-old Tianfu green shell laying hens from the Chicken Breeding Unit at Sichuan Agricultural University were used in this study. The chickens were randomly divided into 2 groups, including the experimental group (n = 12) and the control group (n = 12). For experimental group, the hens were subcutaneously injected with TMX (estrogen receptor modulator) (Sigma, St. Louis, MO) at a dose of 6 mg/0.3 mL ethanol/kg per body weight; however, the control group chickens were treated with ethanol, as previous described (Arao et al., 1996; Wolak and Hrabia, 2020). The hens were injected with TMX or ethanol daily (in the morning) until egg production of all TMX-treated chickens was suspended (Socha et al., 2018). Egg laying rate of the hens in the experimental group and control group were recorded daily. All hens were euthanized on day 6 of the experiment, and the ovaries were collected and weighed, meanwhile, fat, spleen, brain, ovary, lung, heart, skeletal muscle, gizzard, liver tissues/organs were collected from the experimental and control group. The samples collected were temporary stored in liquid nitrogen and later stored at -80℃ for subsequent RNA extraction.

Chicken Granulosa Cells Isolation and Culture

Chicken granulosa cells were collected and cultured according to our previous study (He et al., 2022; Wei et al., 2022). Briefly, the granular layer was cut into pieces and digested with collagenase type II (BIOFROXX, Einhausen, Germany) for 7 to 10 min before cells were filtered through a 70 μm cell sieve. Subsequently, the granulosa cells were resuspended and plated with Medium 199 (Gibco, Langley) with 10% fetal bovine serum (**FBS**, Gibco, Grand Island, NY) and 1% penicillin/streptomycin (Solarbio, Beijing, China) at 37° C, 5% CO_2 and saturated humidity.

Oligonucleotides, Plasmids Construction, and Transfection

MiR-34a-5p inhibitor, inhibitor NC, miR-34a-5p mimic, Mimic NC, small interfering RNAs (si-LEF1), and siRNA negative control (**si-ctrl**) for LEF1 were designed and synthesized by GenePharma (GenePharma, Shanghai, China). The LEF1 overexpression plasmids (ov-LEF1) were constructed using pcDNA3.1 vector (**ov-ctrl**) (Geneseed Biotech, Guangzhou, China). For the construction of pmirGLO Dual-Luciferase reporter vector, the wild type (LEF1-WT) and mutated sequences (**LEF1-MUT**) of LEF1 were cloned into the pmirGLO Dual-Luciferase reporter vector (Tsingke, Beijing, China). For transfection, lipofectamine 3,000 (Invitrogen, Carlsbad, CA) and Opti-MEM (Gibco) were used for cell transfection according to the manufacturer's instructions (Zhao et al., 2022). First,

siRNA or overexpressed plasmids were mixed with Optim-MEM for 5 min. Then add lipofectamine 3,000 and incubate for 20 min at room temperature. They were then added separately to cell culture plates. After 24 h, the transfection efficiency was detected by quantitative real-time PCR (qPCR) or western blot and further analysis was performed.

RNA Extraction, cDNA Synthesis, and qPCR

Total RNA extraction from follicular tissues and granulosa cells using Trizol Reagent (Invitrogen, Carlsbad, CA). The miRNA qPCR Primer was used to measure the expression levels of miRNAs by qRT−PCRs with SYBR Green Master Mix (TaKaRa, Dalian, China) using a LightCycler 96 according to described previously (Zhao et al., 2022). U6 (for miRNA) were used as the internal control. For mRNA analysis, cDNA was synthesized using the Takara PrimeScript RT reagent kit (Takara), and Takara SYBR Green Master Mix was used for quantitative PCR. β-actin (for mRNA) were used as the internal control. All qPCR primers were designed using Primer Premier 5 (PREMIER Biosoft, CA) and synthesized by Tsingke (Tsingke, Beijing, China), as listed in Table 1. The qPCR results were analyzed using $2^{-\Delta\Delta Ct}$ method. Each sample was assayed in triplicates.

Western Blot

After transfection for 48 h, the medium was removed and washed twice with PBS, the total proteins from the granulosa cells were extracted using Total Protein Tissue or Cell Total Protein Extraction kit (Solarbio, Beijing, China) and the protein concentrations was measured using a bicinchoninic acid (**BCA**) protein assay kit (BestBio, Shanghai, China). Further, equivalent quality of proteins were separated by SDS-polyacrylamide gel electrophoresis (**SDS-PAGE**) and transferred to polyvinylidene fluoride (**PVDF**) membranes. To calculate molecular weight, Kaleidoscope prestained standards (Sigma) were utilized. Subsequently, seal with 5% skim milk, the PVDF membranes were incubated with specific primary antibody, including anti-caspase 3 (Abcam, London, UK, 1:1,000), anti-caspase 8 (ABclonal, Wuhan, China, 1:1,000), anti-LC3 (Cell Signaling Technology, Boston, Mass), anti-Beclin1 (ABclonal, 1:1,000), anti-LEF1 (ABclonal, 1:1,000), and GAPDH (ZenBio, Chengdu, China; 1:5,000). The next day, the primary antibody was recovered and the specific secondary antibody was incubated with the membrane. Finally, the required protein bands were strengthened by the enhanced chemiluminescence (**ECL**) kit (Beyotime, Shanghai, China) and harvested under the Image lab software (National Health Institute, Bethesda, MD). GAPDH was used as the internal reference in this study. The strip density was analyzed using ImageJ software.

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

Gene	Primer sequences (5′→3′)	Length (bp)
miR-34a-5p	F: UGGCAGUGUCUUAGCUGGUUGUU R: CAGGTCCAGTTTTTTTTTTTTTT	/
U6	F: GGGCCATGCTAATCTTCTCTGTA R: CAGGTCCAGTTTTTTTTTTTTTT	/
LEF1	F: GCCACCGACGAGATGA R: TGACCAGCGAGGACTTG	121
caspase 3	F: TGGCCCTCTTGAACTGAAAG R: TCCACTGTCTGCTTCAATACC	139
caspase 8	F: CCCTGAAGACAGTGCCATTT R: GGGTCGGCTGGTCATTTTAT	106
caspase 9	F: GCTTGTCCATCCCAGTCCAA R: CAGTCTGTGGTCGCTCTTGT	95
β-actin	F: GTCCACCGCAAATGCTTCTAA R: TGCGCATTTATGGGTTTTGTT	78

Flow Cytometric Apoptosis Analysis

The granulosa cells were seeded in 6-well plates and transfected 48 h later, cells were collected and stained using Annexin V-FITC apoptosis detection kit (Beyotime, Shanghai, China). Further, the results were obtained by a flow cytometer (Beckman, Miami, FL) following the manufacturers protocol.

Dual Luciferase Report Analysis

Chicken fibroblast cell line (DF-1 cells) was subjected to dual luciferase report analysis. Similarly, the DF-1 cells were incubated in the medium that provides 10% FBS (Gibco) and 1% penicillin/streptomycin (Solarbio) at 37°C in humidified atmosphere of 5% CO_2. Then the DF-1 cells were plated in 48-well plates and then were co-transfected with LEF1-WT or LEF1-MUT and miR-34a-5p mimic or Mimic NC. After transfection for 48 h, the luminescent values of firefly and Renilla luciferase were detected by Dual-GLO Luciferase Assay System Kit (Promega, Madison, WI) according to the manufacturer's protocol.

Double-Labeled Adenovirus mRFP-GFP-LC3 Transfection

Adenovirus was used to process granulosa cells after transfection 24 h. Subsequently, the cells were fixed with 4% paraformaldehyde (Beyotime) for 30 min and then a confocal microscope (Olympus, New York, NY) was used to take randomly pictures following the procedures described previously (Han et al., 2019).

RNA-Seq

Total RNA was extracted from Inhibitor NC and miR-34a-5p inhibitor cells, using a RNeasy Mini Kit (Thermo Fisher Scientific, Waltham, MA) and on-column DNase digestion (Thermo Fisher Scientific, Waltham, MA) to prevent genomic DNA contamination. Biomarker Technologies Co., Ltd. (Beijing, China) constructed the cDNA libraries, carried out the sequencing, and analyzed the transcriptome data, rigorous adherence to the corporate

standard operating procedures, which are posted online (http://www.biomarker.com.cn/).

Statistical Analysis

All the data obtained in this study were analyzed using SPSS 19.0 statistical software (SPSS, Chicago, IL). Two group comparisons were performed using 2-tailed Student's t test and multiple group comparison were analyzed by 1-way ANOVA analysis. GraphPad 8.0 (GraphPad, La Jolla, CA) was used for plotting graphs. All results were expressed as mean ± SEM. The significant level was set at $^{*}P < 0.05$, $^{**}P < 0.01$, and ^{a,b} $P < 0.05$.

RESULTS

The Expression Pattern of miR-34a-5p in Chicken

In this study, we successfully induced follicular atresia in hens by subcutaneous injection of TMX, as shown in Figure 1A, TMX-induced hens completely stopped laying eggs after 6 d of treatment. In addition, TMX treatment also resulted in a significant reduction in the weight of ovaries ($P < 0.01$, Figures 1B and 1C). Furthermore, we found that the expression of miR-34a-5p in the ovaries of TMX-induced hens was approximately twice that of the normal group ($P < 0.01$, Figure 1D). Furthermore, the expression schema of miR-34a-5p in different tissues of chickens was verified by qPCR, the result have shown that the miR-34a-5p was highly expressed in fat tissue, as well as in the ovary ($P < 0.05$, Figure 1E). Finally, we compared the seed sequences of miR-34a-5p in different species (including human, chimp, mouse, rat, pig, and cow) and confirmed that it was a conserved miRNA (Figure 1F). All the data have manifested that the miR-34a-5p was highly expressed in the atretic follicles of hens and had higher conservatism in different species, which implies that miR-34a-5p is essential for the development of chicken follicles.

Effects of miR-34a-5p on Apoptosis of Chicken Follicular Granulosa Cells

First, the expression of miR-34a-5p was successfully lowered and elevated by the treatment of miR-34a-5p inhibitor and miR-34a-5p mimic in chicken granulosa cells ($P < 0.01$, Figures 2A and 2B). Moreover, qPCR results found that the mRNA level of caspase 3, caspase 8, and caspase 9 were obviously down-regulated in the miR-34a-5p inhibitor transfected group ($P < 0.05$, Figure 2C); however, the exogenous miR-34a-5p increased 3 apoptosis-related genes expression ($P < 0.05$, Figure 2D). The flow cytometric apoptosis analysis revealed that interference of miR-34a-5p visibly decreased the amounts of apoptotic cells ($P < 0.05$, Figure 2E), and the over-expression of miR-34a-5p exacerbated the proportion of apoptosis ($P < 0.01$, Figure 2F). Similar results were also shown in the western blot, which showed that the inhibitor miR-34a-5p hindered the protein expression of apoptosis-related genes ($P < 0.01$, Figure 2G), whereas overexpression miR-34a-5p showed an opposite effect ($P < 0.05$, Figure 2H). These results indicated that miR-34a-5p accelerates the apoptosis of chicken granulosa cells.

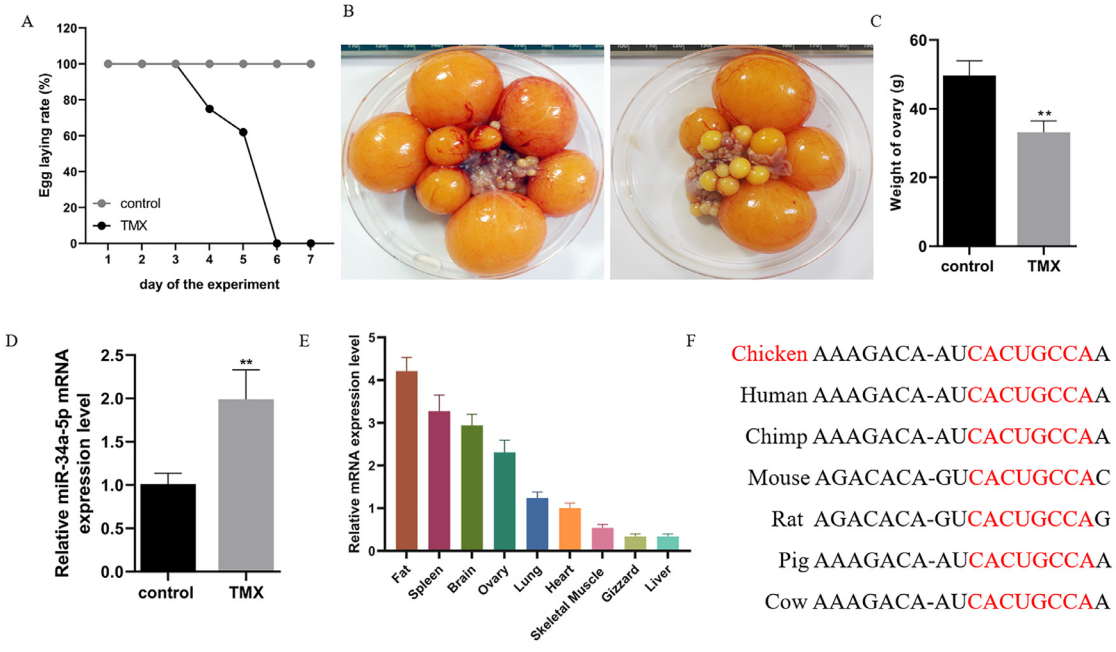

Figure 1. The expression pattern of miR-34a-5p in chicken. (A) Egg-laying rate (B) ovary morphology (C) ovary weight induced by tamoxifen (TMX) in laying hens (n = 12). (D) The expression of miR-34a-5p in control and TMX treatment ovary (n = 3). (E) The expression of miR-34a-5p in different tissues and organs of chicken (n = 3). (F) Seed region of miR-34a-5p in different species (n = 3). Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

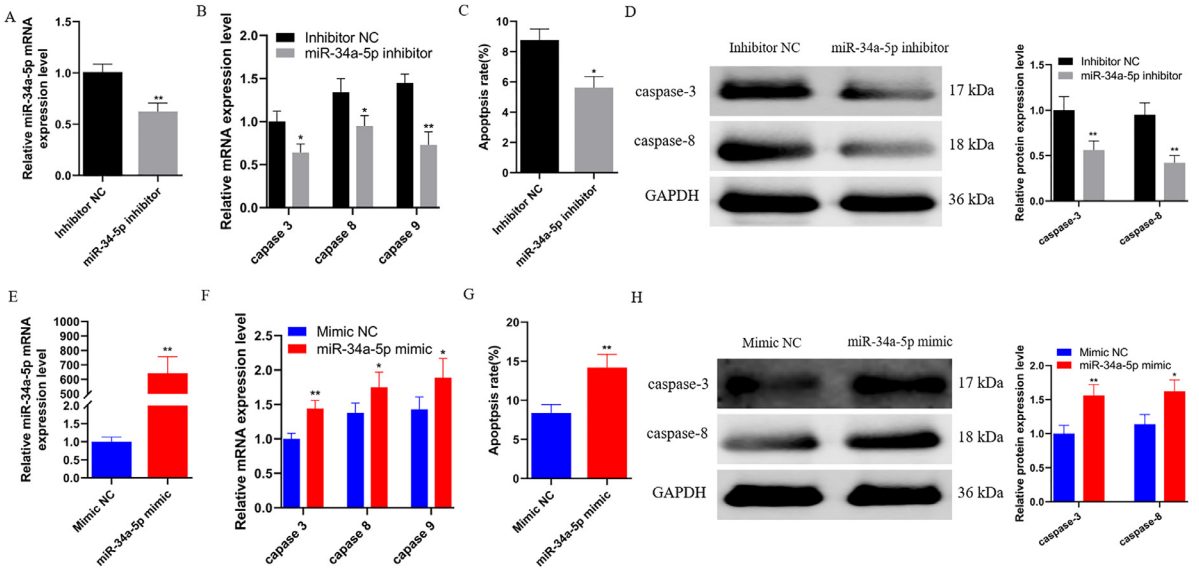

Figure 2. Effects of miR-34a-5p on apoptosis of chicken follicular granulosa cells. (A) The expression of miR-34a-5p after knockdown of miR-34a-5p. (B) The expression of miR-34a-5p after overexpression of miR-34a-5p. (C) The mRNA level of caspase 3, caspase 8, and caspase 9 after knockdown of miR-34a-5p. (D) The mRNA level of caspase 3, caspase 8, and caspase 9 after overexpression of miR-34a-5p. (E) The apoptosis ratio after knockdown of miR-34a-5p. (F) The apoptosis ratio after overexpression of miR-34a-5p. (G) The protein level of caspase 3 and caspase 8 after knockdown of miR-34a-5p. (H) The protein level of caspase 3 and caspase 8 after overexpression of miR-34a-5p. Data are expressed as mean ± SEM (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$.

Effects of miR-34a-5p on Autophagy of Chicken Follicular Granulosa Cells

To explore the impact of miR-34a-5p on chicken granulosa cells autophagy, mRFP-GFP-LC3 was conducted and applied to assess the degree of autophagy flux. As shown in Figures 3A and 3B ($P < 0.05$), autophagic flux was clearly reduced after interference of miR-34a-5p. Western blot results indicated that the proteins levels of the autophagy related genes, such as LC3Ⅱ and Beclin1 were reduced in miR-34a-5p inhibitor transfected group ($P < 0.05$, Figure 3C). On the contrary, overexpression of miR-34a-5p accelerated the degree of autophagy flux ($P < 0.01$, Figures 3D and 3E), and the level of LC3Ⅱ and Beclin1 proteins were significantly increased ($P <$ 0.05, Figure 3F). Taken together, these results confirmed that miR-34a-5p facilitated the autophagy of chicken granulosa cells.

miR-34a-5p Affects Apoptosis of Chicken Follicular Granulosa Cells by Mediating Autophagy

To further explore the role of miR-34a-5p on autophagy and apoptosis, we treated the granulosa cells with autophagy inhibitor 3-Methyladenine (**3MA**) after transfection of miR-34a-5p mimic and Mimic NC, and the results showed that 3MA significantly reduced the level of autophagy of the granulosa cells ($P < 0.05$,

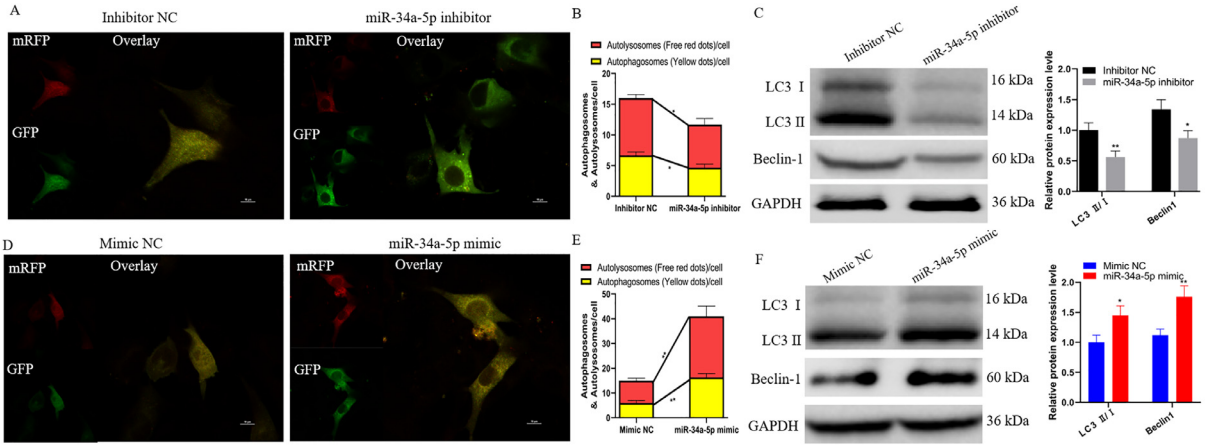

Figure 3. Effects of miR-34a-5p on autophagy of chicken follicular granulosa cells. (A, B) The autophagosomes fluorescence picture after interference of miR-34a-5p. Bar scale = 10 μm. (Green fluorescence: lysosome; Red fluorescence: Autolysosomes; Yellow fluorescence: Autophagosomes). (C) The protein abundant of LC3 and Beclin1 after interference of miR-34a-5p. (D, E) The autophagosomes fluorescence picture after overexpression of miR-34a-5p. (F) The protein abundant of LC3 and Beclin1 after overexpression of miR-34a-5p. Data are expressed as mean ± SEM (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$.

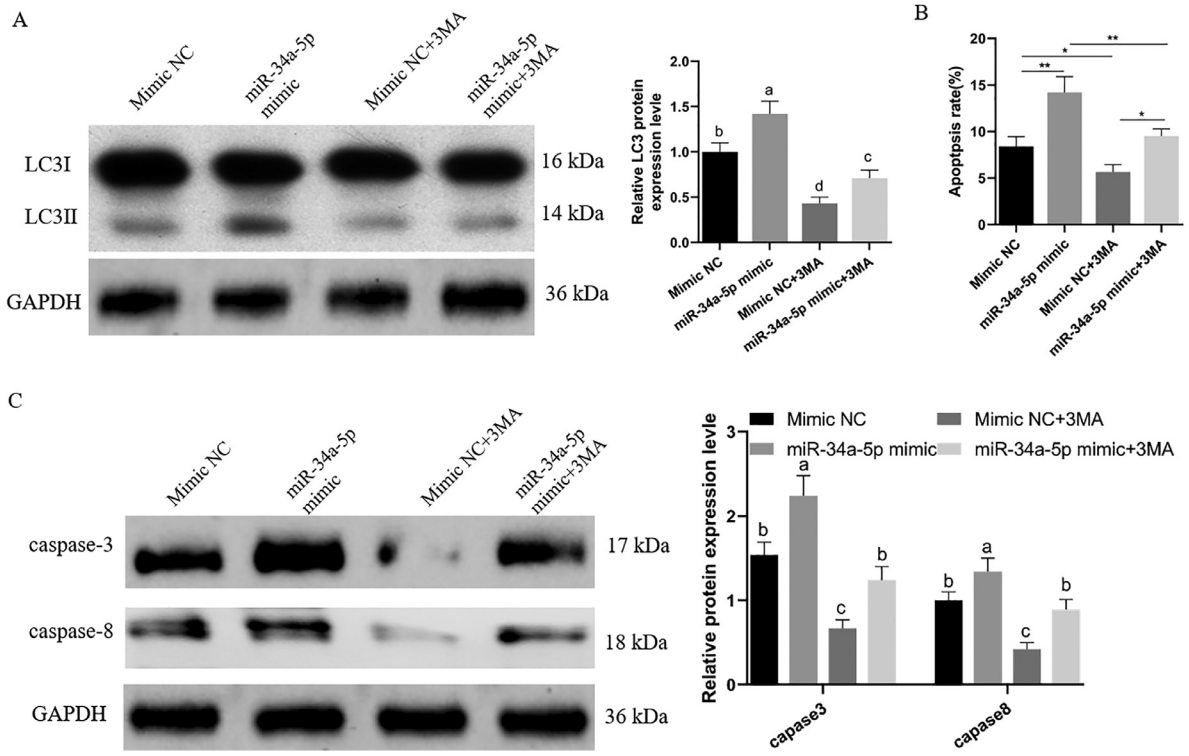

Figure 4. miR-34a-5p affects apoptosis of chicken follicular granulosa cells by mediating autophagy. (A) The protein levels of LC3 after 3MA treatment of Mimic NC and miR-34a-5p mimic transfected cells. (B) The apoptosis ratio of after 3MA treatment of Mimic NC and miR-34a-5p mimic transfected cells. (C) The protein level of caspase 3, caspase 8 after 3MA treatment of Mimic NC and miR-34a-5p mimic transfected cells. Data are expressed as mean ± SEM (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$ and ^{a,b} $P < 0.05$.

Figure 4A). Subsequently, the rate of apoptosis was detected after 3MA treatment of transfected cells, the results have shown that the 3MA treatment gave a decrease tendency in cell apoptosis levels compared to the mimic group ($P < 0.05$, Figure 4B). Moreover, the protein expression of the apoptosis-related genes changed synchronously with LC3 protein level after 3MA treatment ($P < 0.05$, Figure 4C). This showed that the miR-34a-5p promotes apoptosis of chicken granulosa cells by mediating autophagy.

LEF1 Is a Direct Target of miR-34a-5p in Follicular Granulosa Cells

To understand the potential mechanisms of miR-34a-5p regulated follicle development, TargetScan website (http://www.targetscan.org/vert_71/) was applied to forecast target gene for miR-34a-5p, and the results indicated that LEF1 was a candidate target gene for miR-34a-5p. To confirm this result, we constructed LEF1-WT and LEF1-MUT vector for dual luciferase report assay (Figure 5A). The luciferase analysis suggested that the exogenous miR-34a-5p obviously reduced the luciferase activity in the DF-cells transfected with the LEF1-WT; however, it made no difference for the luciferase activity of the LEF1-MUT ($P < 0.05$, Figure 5B). Besides, the qPCR results have revealed that the miR-34-5p was knock-downed in favor of mRNA level of LEF1; however, the overexpression of miR-34a-5p has showed an opposite effect ($P < 0.05$, Figure 5C). Finally, the results of the level of LEF1 protein were consistent with those of qPCR ($P < 0.01$, Figures 5D and 5E). These results have indicated miR-34a-5p targets LEF1 to perform regulatory functions in follicular granulosa cells.

Effects of LEF1 on Autophagy and Apoptosis of Chicken Follicular Granulosa Cells

To verify the role of LEF1 in chicken granulosa cells, we successfully reduced the mRNA level of LEF1 by transfecting si-LEF1 ($P < 0.01$, Figure 6B). Subsequently, the protein levels of LC3Ⅱ and Beclin1 were significantly up-regulated in the si-LEF1 transfected cells ($P < 0.01$, Figure 6A). In addition, the knockdown of LEF1 markedly augmented the mRNA levels of caspase 3, caspase 8, and caspase 9 ($P < 0.05$, Figure 6C). The flow cytometric apoptosis analysis revealed that the interference of LEF1 accelerated the apoptosis of chicken granulosa cells ($P < 0.01$, Figure 6D). Inversely, we overexpressed LEF1 by transfecting ov-LEF1 in the chicken granulosa cells ($P < 0.01$, Figure 6F). Moreover, the overexpression of LEF1 observably decreased the degree of autophagy ($P < 0.05$, Figure 6E) and apoptosis rate ($P < 0.05$, Figures 6G and 6H). These results showed that LEF1, in contrast to miR-34a-5p, could relieve autophagy and apoptosis of chicken granulosa cells.

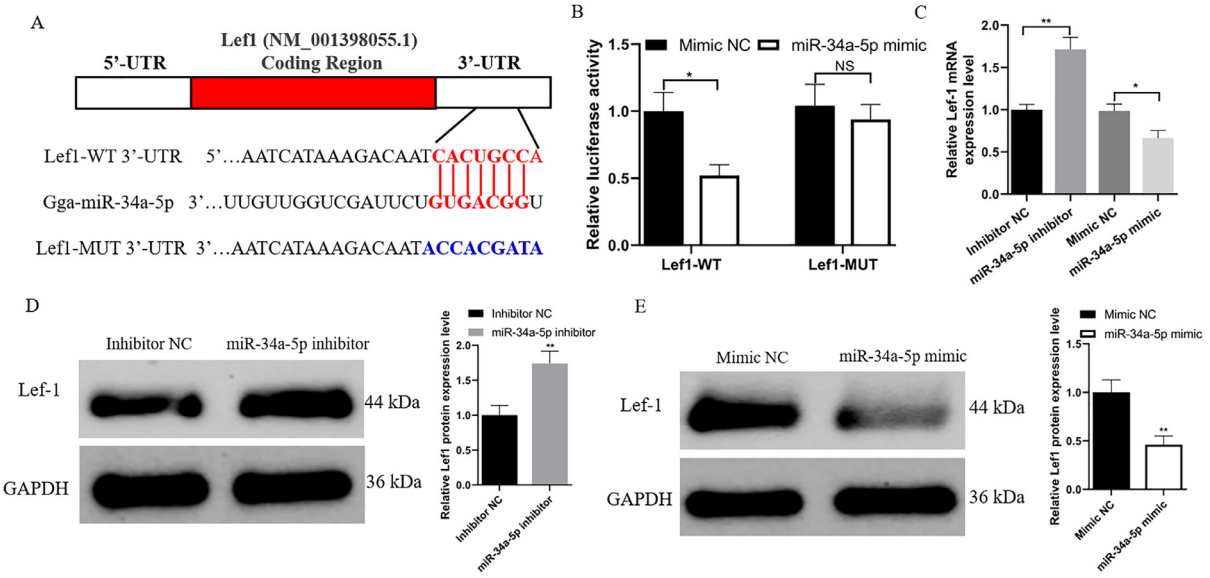

Figure 5. LEF1 is a direct target of miR-34a-5p in follicular granulosa cells. (A) Construction of wild-type dual-luciferase reporter gene and mutant dual-luciferase reporter gene of LEF1. The seed sequences are highlighted in red and the mutant sequences are highlight in blue. (B) The dual-luciferase reporter assay was performed after co-transfected with LEF1-WT or LEF1-MUT and miR-34a-5p mimic or Mimic NC. (C) The mRNA level of LEF1 after knockdown and overexpression of miR-34a-5p. (D, F) The protein level of LEF1 after knockdown and overexpression of miR-34a-5p. Data are expressed as mean ± SEM (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$.

miR-34a-5p Mediates the Hippo-YAP Signaling Pathway by Targeting LEF1

Kyoto Encyclopedia of Genes and Genomes (**KEGG**) enrichment analysis of the target genes of miR-34a-5p were performed, and the results have indicated that the target genes were mainly related to the Hippo signaling pathway and peroxisome proliferator activated receptor (**PPAR**) signaling pathway (Figure 7A). The Hippo-YAP pathway is essential for ovarian development (Hall et al., 2010; Kawamura et al., 2013; Cheng et al., 2015). In order to evaluate that miR-34a-5p preformed regulatory role through mediating the Hippo signaling pathway, we detected the gene expression of core effectors such as large tumor suppressor kinase 1 (**LATS1**) and Yes1 associated transcriptional regulator (**YAP**) of the Hippo-YAP signaling pathway, the results shown that the protein levels of LATS1, YAP, and p-YAP were significantly decreased in the miR-34a-5p inhibitor cells ($P < 0.05$, Figure 7B), whereas overexpression of miR-34a-5p promoted the protein level of LATS1, YAP, and p-YAP ($P < 0.05$, Figure 7C). In addition, we found that

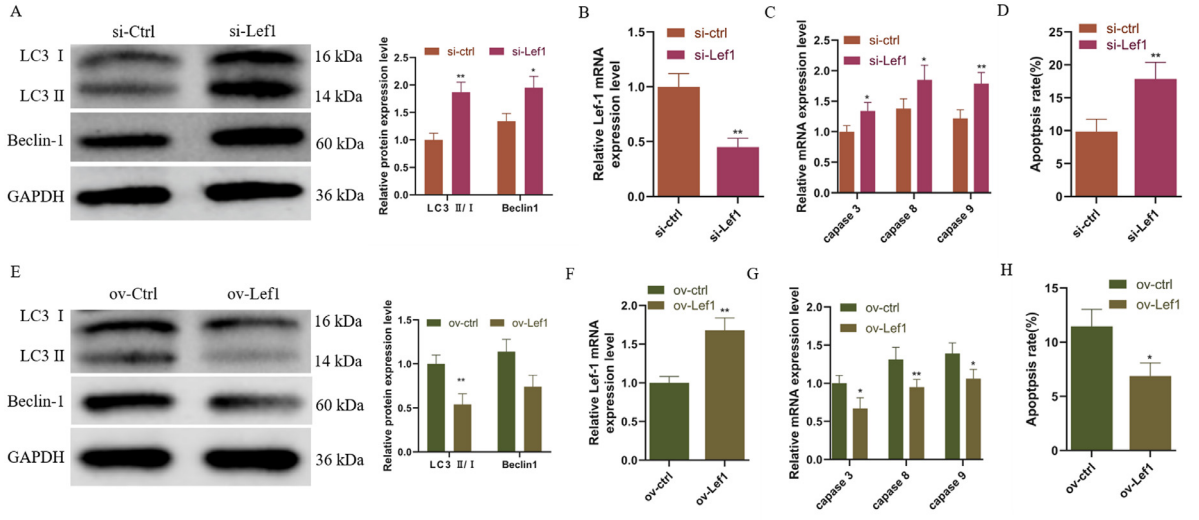

Figure 6. Effects of LEF1 on autophagy and apoptosis of chicken follicular granulosa cells. (A) The protein level of LC3 and Beclin1 after interference of LEF1. (B) The mRNA level of LEF1 in si-LEF1 and si-ctrl cells. (C) The mRNA expression of caspase 3, caspase 8 and caspase 9 in si-LEF1 and si-ctrl cells. (D) The cell apoptosis ratio after knockdown of LEF1. (E) The protein level of LC3 and Beclin1 after overexpression of LEF1. (F) The mRNA level of LEF1 in ov-LEF1 and ov-ctrl cells. (G) The mRNA expression of caspase 3, caspase 8 and caspase 9 in ov-LEF1 and ov-ctrl cells. (H) The cell apoptosis ratio after overexpression of LEF1. Data are expressed as mean ± SEM (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$.

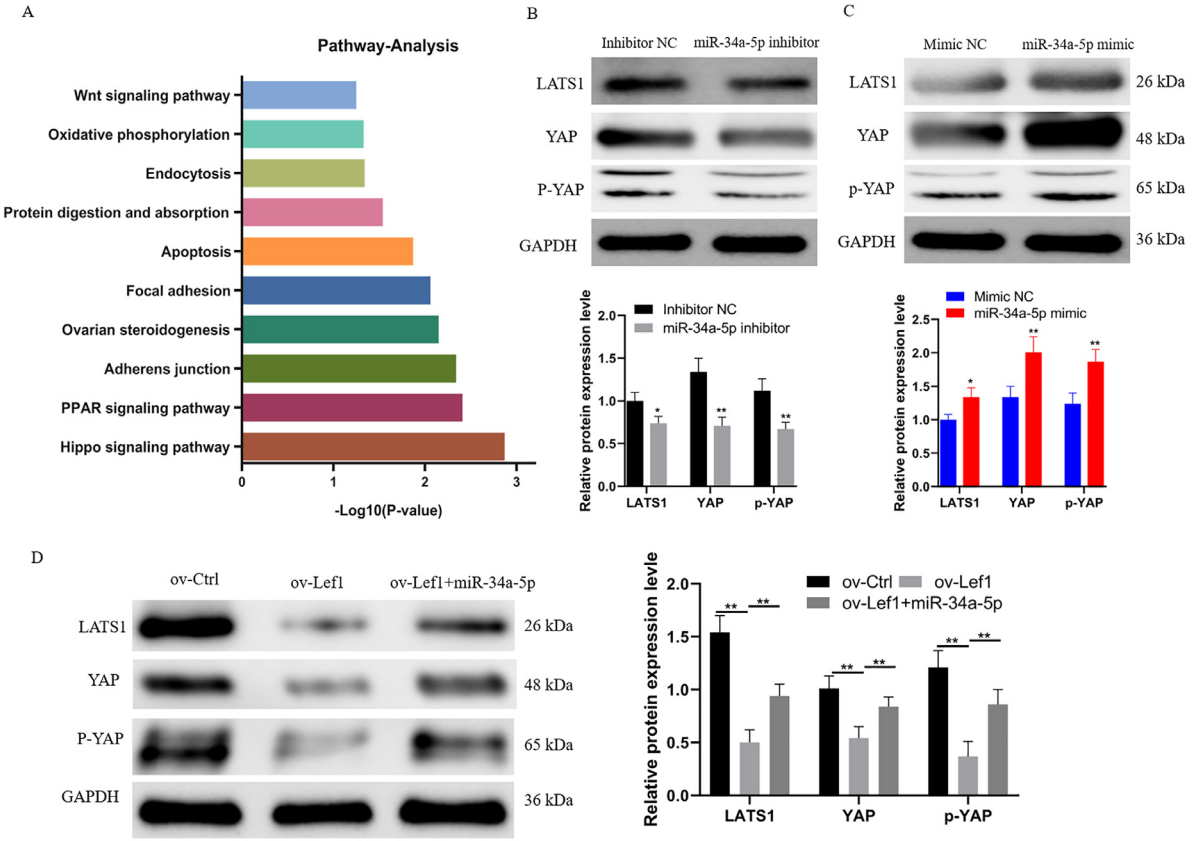

Figure 7. miR-34a-5p mediates the Hippo-YAP signaling pathway by targeting LEF1. (A) KEGG analyses of the target genes of miR-34a-5p. (B) Relative protein level of the Hippo-YAP signaling pathway after interference of miR-34a-5p. (C) Relative protein level of the Hippo-YAP signaling pathway after overexpression of miR-34a-5p. (D) Relative protein level of the Hippo-YAP signaling pathway after overexpression of Lef1 or Lef1 +miR-34a-5p. Data are expressed as mean ± SEM (n = 3). $^{*}P < 0.05$; $^{**}P < 0.01$.

the Hippo pathway was inhibited by overexpression of LEF1, while the Hippo pathway was activated by co-transfection of LEF1and miR-34a-5p ($P < 0.05$, Figure 7D). These results indicated that miR-34a-5p activated the Hippo-YAP signaling pathway to regulate chicken follicular development by targeting LEF1.

DISCUSSION

Follicular atresia is a necessary and normal physiological phenomenon in poultry reproduction. Currently, it has been reported that apoptosis of granulosa cells is the fundamental cause of follicular atresia (Zhang et al.,

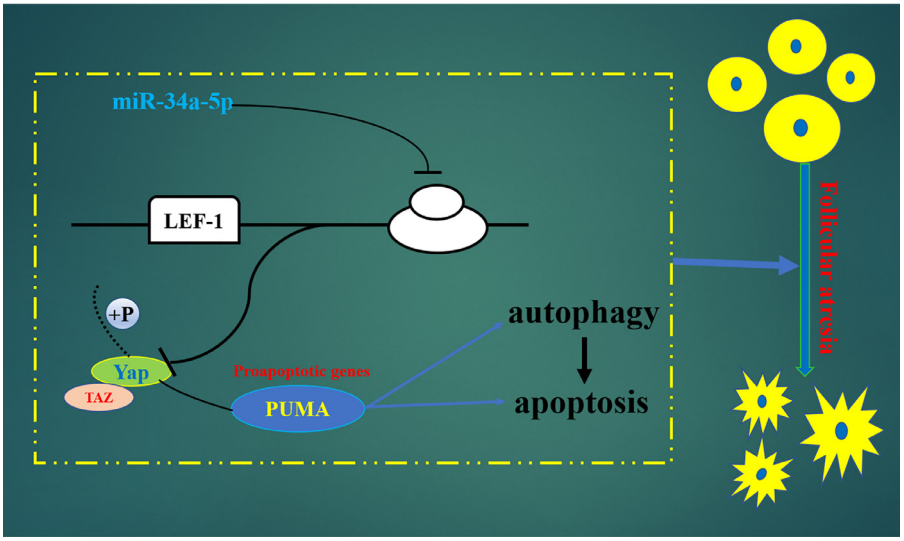

Figure 8. Model diagram of miR-34a-5p target LEF1 to regulate follicular atresia via mediating the Hippo-YAP signaling pathway.

2019). However, recent studies have found that follicle atresia is regulated by both autophagy and apoptosis in poultry (Yamamoto et al., 2015). Therefore, it is necessary to explore and verify the regulatory mechanisms of autophagy and apoptosis on follicular atresia for poultry egg production.

Numerous human and animal studies have revealed that miRNAs play key role in ovarian development by regulating gene expression after transcription (Zhang et al., 2019). Importantly, miRNAs were involved in follicular atresia by mediating autophagy and apoptosis of granulosa cells. For example, a study indicated that miR-141-3p directly targeted death associated protein kinase 1 (**DAPK1**) to hamper apoptosis of ovarian granulosa in rat (Li et al., 2017). MiR-150 via regulating steroidogenic acute regulatory protein (**STAR**) gene to promote ovine ovarian granulosa cells apoptosis (Zhou et al., 2019). In our previous study, we conducted deep miRNA sequencing of healthy and atretic follicles of chicken and found that miR-30a-5p was highly expressed in atretic follicles, and also revealed that miR-30a-5p inhibited autophagy and apoptosis of chicken granulosa cells by targeting Beclin1 (He et al., 2022). Although the potential functions of numerous miRNAs for ovarian development in different species have been explored, the function of miR-34a-5p in follicular atresia of chickens remains unclear. In this study, we deeply explored the functions of miR-34a-5p in chicken follicular granulosa cells, and the results shown that miR-34a-5p contributes to granulosa cells death by facilitating autophagy and apoptosis.

In the present study, we confirmed that LEF1 acts as a target gene for miR-34a-5p. LEF1, a component of canonical Wnt-signaling, is related to the procession of cell cycle, autophagy and apoptosis in different types of cells (Gao et al., 2014). Besides, LEF1 is highly expressed in the ovarian surface epithelium stem cells (Flesken-Nikitin et al., 2013) and could be considered as an auxiliary tab for examining of tubal lesions (Schmoeckel et al., 2017). However, there are no reports that reveal the effect of LEF1 in chicken granulosa cells. Subsequently, we investigated that the effects of LEF1 on autophagy and apoptosis during follicular atresia in chickens, the results that LEF1 has the opposite effect to miR-34a-5p, which could alleviate autophagy and apoptosis in chicken follicular atresia. Moreover, LEF1 is a key target for molecular docking in the Hippo signaling pathway (Zhang et al., 2019). The Hippo signaling pathway is not only took part in cell proliferation and apoptosis, but also regarded as a regulator of autophagy (Jia et al., 2003; Pavel et al., 2021). It was reported that pyruvate kinase M1/2 (PKM) controlled the proteins level of cell apoptosis and proliferation-related genes by activating Hippo signaling pathway (Luo et al., 2021). MiR-9 was found to promote apoptosis in human acute myeloid leukemia cells by regulating the Hippo-YAP signaling pathway (Wang et al., 2021). P62/SQSTM1 co-located with the hippocampal signaling modulator Dachs and inactivated the Hippo signaling pathway, leading to excessive non-cellular proliferation of stem cells (Nagai et al., 2021). The Hippo-YAP pathway also manipulated proliferation and autophagy in lung adenocarcinomas (Xu et al., 2021). In present experiment, we confirmed that miR-34a-5p regulates autophagy and apoptosis by targeting LEF1 to mediate the Hippo-YAP signaling pathway in chicken granulosa cells. Taken together, the results obtained in this present study have implied that miR-34a-5p plays a vital role in follicular atresia via mediating autophagy and apoptosis.

CONCLUSION

In summary, as shown in Figure 8, our results revealed that miR-34a-5p promotes autophagy and apoptosis of chicken follicular granulosa cells by targeting LEF1 to mediate the Hippo-YAP signaling pathway, suggesting that miR-34a-5p is indispensable for chicken follicular atresia. Therefore, the results may provide theoretical basis for the utilization and exploitation of egg laying performance in poultry.

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DISCLOSURES

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

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