

# The relationship between the mTOR signaling pathway and ovarian aging in peak-phase and late-phase laying hens

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**ABSTRACT** The molecular mechanisms underlying reproductive aging in avian species are poorly understood. Previous studies have shown the importance of mechanistic target of rapamycin (**mTOR**) signaling pathway in aging. In this study, we investigated the relationship between the mTOR signaling pathway and ovarian aging in the peak phase and late phase of egg production in laying hens. The egg production rate and egg quality were tracked for 5 consecutive weeks in 30-week-old and 70-week-old Dawu Jinfeng hens (N = 30/group). During the peak phase (week 35) and late phase (week 75), antioxidant and immune indicators were detected by enzyme-linked immunosorbent assay, and mTOR signaling-related genes (CLIP-170, GRB10, LIPIN-1, ATG1, 4E-BP1, S6K, PKC, RHO, and SGK1) were detected in the follicles by quantitative reverse transcription-PCR technology. The protein expression of key genes (mTOR, PKC, 4EBP1) was evaluated by Western blot analysis. The egg production rate and the antioxidant indexes superoxide dismutase and glutathione peroxidase and the levels of total antioxidant capacity and immunoglobulins (IgM and IgG) were significantly higher at week 35 than those at week 75 ( $P < 0.01$ ), while malondialdehyde levels were significantly lower

( $P < 0.01$ ). At week 75, there were fewer follicles in the different stages of development than were detected at week 35. The number of white follicles (large and small) and primary follicles were significantly higher at week 75 than those detected at week 35 ( $P < 0.01$ ). The mRNA expression of *avTOR*, *CLIP-170*, *GRB10*, *LIPIN-1*, *4E-BP1*, *S6K*, *RHO*, and *SGK* genes in small white follicles (**SWF**), large white follicles (**LWF**), F3, F1, and ovary at week 75 was lower than that in the hens at week 35 ( $P < 0.05$ ). The mRNA expression in small yellow follicle (**SYF**) was significantly higher than that at week 35 ( $P < 0.05$ ), while the mRNA expression of *ULK1* in SWF, LWF, F3, F1, and ovary at week 75 was higher than that of hens at week 35 ( $P < 0.01$ ), and SYF was lower ( $P < 0.05$ ). Treatment of chicken granulosa cells with the mTOR agonist MHY1485 significantly enhanced granulocyte proliferation ( $P < 0.01$ ) and inhibited apoptosis ( $P < 0.01$ ) and significantly increased *avTOR*, *S6K*, *4E-BP1*, and *PKC* gene expression ( $P < 0.01$ ). The protein expression levels of mTOR, S6K, p-mTOR, and p-S6K were consistent with mRNA expression levels. The mTOR activity is age-specific, and a compensatory enhancement of the mTOR signaling cascade can regulate ovarian follicular development in aged laying hens.

**Key words:** mTOR, aging, follicle, peak-phase laying hen, late-phase laying hen

2021 Poultry Science 100:334–347

<https://doi.org/10.1016/j.psj.2020.10.005>

## INTRODUCTION

Aging is a normal but complex and irreversible biological process that is common to all living organisms (Martin, 2011). Organ, tissue, and cell functions decline with aging. Furthermore, the rate of errors in DNA transcription, translation, and protein synthesis gradually

increases with aging, while repair function gradually decreases. Aging may be an important factor that induces the aging of other organs (Marx, 2008), leading to an increased risk of various diseases, such as osteoporosis (Li and Wang, 2018), tumors, and cardiovascular disease (Eyster and Brannian, 2009; Morozova et al., 2011; Collins et al., 2016). Studies have shown that ovarian aging occurs earlier than in other tissues (Zhang et al., 2019). During aging, the volume and weight of the reproductive organs decrease and are accompanied by a decline in the levels of secreted reproductive hormones (Gary and Feldman, 1991; Morley et al., 1997; Mulligan et al., 1997; Ferrini and Barrett-Connor, 1998). In recent years, reproductive aging has received

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Received April 24, 2020.

Accepted October 6, 2020.

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increasing attention, with age-related ovarian insufficiency as the main basis of reproductive failure in women. Therefore, finding the main cause of reproductive aging is of great significance in delaying this process.

The mechanistic target of rapamycin (**mTOR**) is a serine/threonine protein kinase that functions as a master regulator of cellular growth and metabolism in response to nutritional and hormonal cues that are closely related to cell growth and development. mTOR consists of 2 distinct complexes, mTORC1 and mTORC2. Pharmacological inhibition of TORC1 signaling extends lifespan in yeast and mice (Powers et al., 2006; Wilkinson et al., 2012). Rapamycin, for example, delays the onset of age-related diseases and extends lifespan (Harrison et al., 2009; Miller et al., 2011). In recent years, mTOR and its related signaling pathway have become a potential target for female fertility protection.

In the present study, we hypothesized that ovarian aging in laying hens is associated with decreased mTOR activity in the late laying period, resulting during delayed follicular development and reduced egg production. A comprehensive understanding of the mechanism by which mTOR regulates follicular development will provide new ideas for delaying ovarian aging in laying hens.

## MATERIALS AND METHODS

### Ethics Statement

Practices regarding the care and use of animals for research purposes were in accordance with the institutional and national guidelines and approved by the Animal Use and Ethics Committee of the Agricultural University of Hebei (China). Every effort was made to minimize animal pain, suffering, and distress.

### Animals and Experimental Design

A total of 60 Dawu Jinfeng commercial laying hens were selected at the age of 30 wk ( $N = 30$ ) and 70 wk ( $N = 30$ ) from Breeding Poultry of Hebei Dawu Group, Baoding, China, for use in the present study. All animals were maintained in individual cages (400 mm  $\times$  380 mm  $\times$  350 mm). The animals were housed under standard conditions of temperature ( $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ), relative humidity ( $65 \pm 10\%$ ), and light (16:8 h light/dark cycle). Water and diets were available ad libitum. The egg number and laying rate were monitored to 35 wk and 75 wk in 2 separate groups. All hens were then euthanized 3 h after egg laying.

### Laying Rate and Egg Quality Determination

The laying rate was recorded every day and determined on a weekly basis during the 5 consecutive weeks of the experimental period. At week 35 and week 75, 30 eggs were selected from each group for evaluation of egg quality. Eggs were collected at 4 pm every day for egg quality testing. The eggshell strength and thickness were assessed

for all eggs collected during 5 consecutive weeks. Eggshell strength was measured using the Egg Force Reader (EFR-01, Orka Food Technology Ltd., Bountiful, UT). After removing the inner shell membrane, eggshell thickness was measured using a Vernier caliper.

### Determination of SOD, GSH-Px, T-AOC, MDA, IgA, IgG, and IgM Concentrations in Plasma by Enzyme-Linked Immunosorbent Assay (ELISA)

At 35 wk and 75 wk of age, blood samples (5 mL) were collected from the wing vein of every chicken. Blood samples were centrifuged at 3,000 r/min for 15 min to separate the plasma, which was stored at  $-20^{\circ}\text{C}$  for analyses of blood parameters. Levels of superoxide dismutase (**SOD**), glutathione peroxidase (**GSH-Px**), total antioxidant capacity (**T-AOC**), malondialdehyde (**MDA**), as well as immunoglobulins A, G, and M (**IgA**, **IgG**, and **IgM**) were measured by ELISA using commercial kits (Shanghai Jianglai Biotechnology Co., Ltd., Shanghai, China) in accordance with the manufacturer's instructions. The assay limits of detection, limits of quantification, intraassay coefficients of variation (%), and interassay coefficients of variation (%) were as follows: SOD (0.1 ng/mL, 0.15 ng/mL, 10%, 15%), GSH-Px (0.1 ng/mL, 0.25 ng/mL, 10%, 15%), T-AOC (0.1 U/mL, 0.75 U/mL, 10%, 15%), MDA (0.1 nmol/mL, 0.375 nmol/mL, 10%, 15%), IgA (1.0  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$ , 10%, 15%), IgG (1.0  $\mu\text{g/mL}$ , 25  $\mu\text{g/mL}$ , 10%, 15%), and IgM (1.0  $\mu\text{g/mL}$ , 10  $\mu\text{g/mL}$ , 10%, 15%), respectively.

### Ovarian Follicle Counting

At 35 wk and 75 wk of age, 10 chickens randomly selected from each group were euthanized by cervical dislocation, and the ovaries were removed. The number and size (diameter) of the follicles were recorded. Follicle sizes were determined based on the following parameters: 1) preovulatory follicle (**POF**):  $>10$  mm; 2) small yellow follicle (**SYF**): 8 to 10 mm; 3) large white follicle (**LWF**): 6 to 8 mm; 4) medium white follicle (**MWF**): 4 to 6 mm; 5) small white follicle (**SWF**): 2 to 4 mm; 6) primary follicle:  $\leq 2$  mm (Johnson, 2015).

### Hematoxylin-Eosin Staining

The POF, SYF, LWF, and SWF follicles from 10 hens from each group were used for hematoxylin-eosin (**HE**) staining, with 3 replicates for each type of follicle. Follicles were fixed in 10% buffered formaldehyde, dehydrated in alcohol, cleared in xylol, and embedded in paraffin. The paraffin-embedded follicles were sequentially sectioned (5- $\mu\text{m}$  thickness) using a semiautomatic microtome (RM2245; Leica Biosystems Newcastle Ltd., Newcastle upon Tyne, UK). For all follicles, one of every 6 sections was selected and placed on a standard histological slide. After drying at  $50^{\circ}\text{C}$ , the slides were stained

with HE and mounted with coverslips and synthetic resin (Sigma Chemical Company, St. Louis, MO). Images of the follicle sections were captured by a Moticam 5.0 digital camera (Motic, Hong Kong, China) coupled to a Nikon Eclipse E200 microscope (Nikon Corporation, Tokyo, Japan) using 100× and 400× magnification lenses. Follicular classification and counting were performed using images obtained under 400× magnification lenses. The thicknesses of the follicle granular layer, the inner and outer membrane layers of the follicle, and the connective tissue layer were measured at different stages of follicular development.

## RNA Extraction and Real-Time PCR Analysis

At 35 wk and 75 wk of age, 10 chickens from each group were euthanized. The ovaries were immediately removed, and granular layers were separated from different grade follicles and frozen at  $-80^{\circ}\text{C}$ . Expression levels of *TOR*, *CLIP*, *GRB10*, *LIPIN1*, *ULK1*, *4E-BP1*, *S6K*, *RHO*, *SGK1*, and *PKC* genes in the different follicle tissue samples were measured using real-time reverse transcription PCR (Roche, Light Cycler 480II; Basel, Switzerland). Total RNA was isolated from ovaries and follicles using a total RNA extraction kit (Invitrogen, 12183-555) in accordance with the manufacturer's instructions. The quality and concentration of the extracted RNA were determined by agarose gel electrophoresis and nucleic acid quantification, respectively. The latter was performed using a nucleic acid quantification analyzer (Smart Spec Plus BIO-RAD, CA). Total cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix (Invitrogen, 11752-050; Carlsbad, CA) in accordance with the manufacturer's instructions. Successful cDNA synthesis was confirmed by PCR amplification of the  $\beta$ -actin amplicon. The synthesized cDNA was amplified in a 20- $\mu\text{L}$  PCR reaction

system containing 1  $\mu\text{L}$  of cDNA, 10  $\mu\text{L}$  of Power SYBR Green PCR Master Mix (Roche, 4913914001, CH, GER), 0.5  $\mu\text{L}$  PCR forward primer, 0.5  $\mu\text{L}$  PCR reverse primer (Huada Biological Engineering Technology & Service, Beijing, China), and 8  $\mu\text{L}$  ddH<sub>2</sub>O. PCR products were verified by 1% agarose gel electrophoresis and subsequent DNA sequencing. Standard curves were generated using pooled cDNA. The sequences of primers for qPCR are listed in Table 1. The relative expression level of each gene was calculated based on triplicate samples using the  $2^{-\Delta\Delta\text{Ct}}$  method (Livak and Schmittgen, 2001).

## Western Blot Analysis

At week 35 and week 75, 10 chickens from each group were euthanized. Total protein from the ovary lysates and granular layers of different grade follicles of hens was extracted using T-PER tissue protein extraction reagent (Thermo Pierce, 78510) containing a protease inhibitor cocktail (Thermo Pierce, 78440). Protein concentrations were evaluated using the BCA Protein Assay (Angle Gene Technologies, VB0002, CHN). Briefly, 20  $\mu\text{g}$  of protein lysate was loaded into each lane, and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% Tris-HCl gels. After electrophoresis, the proteins were electroblotted onto a PVDF membrane (Merck Millipore, IPVH00010). Membranes were blocked with Tris-buffered saline (TBS) containing 5% nonfat milk for 1 h at room temperature and were then incubated overnight at  $4^{\circ}\text{C}$  with primary antibodies for the detection of mTOR (CST, 2972, 1:1,000 dilution), 4E-BP1 (CST, 9452, 1:1,000 dilution), PKC (Abcam, ab31, 1:500 dilution), and  $\beta$ -actin (Abcam, ab8226, 1:1,000), which was used for an internal control. Membranes were washed in TBS-T for 15 min to remove unbound primary antibodies (Cell Signaling

**Table 1.** Primers list.

Gene	GenBank accession	Primer sequences (5'-3')	Size (bp)
<i>GRB10</i>	NM_001039282.2	GGGTAGCCAAACTCCACTCC CCATCATCCTCACACGGCAA	237
<i>SGK1</i>	NNM_204476.1	GGGTCCCGCTTTGACTTACTC GAAAGTCCGATGGCTTGGCA	272
<i>ULK1</i>	XM_415091.5	TGATGCCAAACATCCCCAGG GCTGCTGCATCTCTCCAAGA	255
<i>4EBP1</i>	XM_424384	GCGAATGTAGGTGAAGAAGAG AACAGGAAGGCACTCAAGG	146
<i>S6K1</i>	AW_239721	CAATTTGCCTCCCTACCTCA AAGGAGGTTCCACCTTTTCGT	176
<i>TOR</i>	XM_417614	CACAACCACTGCTCGCCACAA CCATAGGATCGCCACACGGATTAG	124
<i>CLIP</i>	NM_204942.1	CCGTACAGGAATCCACAG CTCCCAAGAAACGCACAA	208
<i>PKC</i>	NM_00102804.1	GGCGGACAGGAAGAATACAGAGG GAAGCTGTGTCAGGAATGGTGGTT	146
<i>RHO</i>	NM_001030606.1	GTTCGGCTGGTCACGGTACATC GGTCACTTCTTCTCTGCCTTCTG	238
<i>LIPIN</i>	XM_015276089.1	CCTCACTCTTCACTCAGCAACTCA TCAGGACTGGCAAGTACCTTCAAC	199
<i><math>\beta</math>-actin</i>	XM_424384	GAGAAATTGTGCGTGACATCA CCTGAACCTCTCATTGCCA	152

Technology, Danvers, MA). The membranes were then incubated with goat anti-mouse IgG H&L (HRP) (Abcam, ab6728; Cambridge, 1:5,000 dilution). After washing with 3 times TBS-T for 20 min, protein bands were visualized with ECL reagents (GE, RPN810) and exposure to the Super Signal West Dura Extended Duration Substrate (Thermo Pierce, 34075). The antibodies used in this method were all validated previously for use with chicken samples (Young et al., 2010; Liu et al., 2015; Zhan et al., 2020).

### **Granular Cell Isolation and Culture**

Ten healthy Dawu Jinfeng laying hens aged 35 wk and laying in regular sequences were used in these studies. SYF (diameter 8–10 mm) were placed in a sterile Petri dish filled with PBS buffer. After carefully peeling the outer membrane, a sterile blade was used to cut through the membrane covering the yolk. After removal of the yolk, the remaining follicular membrane layer (basement membrane) and the granular cell layer were washed with PBS. The granular cell layer was carefully removed with tweezers and placed in a centrifuge tube. After washing 3 times with M199, 0.2% type II collagenase was added, and the membrane was incubated at 37°C in a thermostatic water bath and shaken for 30 min. After filtered through a 200-mesh cell sieve, the filtrate was collected and centrifuged at 1,200 rpm for 5 min. The pellet was collected and washed twice with M199 medium, then centrifuged at 1,200 rpm for 5 min. The pellet was resuspended in M199 medium, and the cells were cultured at 37°C under 5% CO<sub>2</sub>. Cells in the exponential growth phase were inoculated into cells culture plates at  $1 \times 10^5$ /mL.

### **MHY1485 Treatment**

Cells treated with culture medium containing 1, 10, and 100 µmol/L mTOR agonist MHY1485 (MCE) were cultured continuously for 96 h; control cells were incubated in normal culture medium. The cells were then maintained at 37°C in 5% CO<sub>2</sub> and 95% air. Cells were harvested 96 h after treatment, and total RNA was isolated using TRIzol reagent (Invitrogen). The RNAs were reverse transcribed by SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). The sequences of primers for qPCR are listed in Table 1. Total protein from chicken granulosa cells was extracted with the Total Extraction kit (Sigma). The procedures described for Western blotting were repeated using primary antibodies against mTOR (Abcam, 1:2,000 dilution), P-mTOR (Abcam, 1:1,500 dilution), S6K (Abcam, 1:1,000 dilution), and p-S6K (Abcam, 1:1,000 dilution) and goat anti-mouse IgG secondary antibody (Abcam, 1:5,000 dilution). An antibody against β-actin (Abcam, 1:1,000 dilution) was used as an internal control. Glyko BandsScan 5.0 (Beijing Sage Creation Science Co., Ltd., Beijing, China) image analysis software was used for densitometric analysis.

### **Cell Proliferation Assay**

Cell proliferation was measured using a cell counting kit-8 cell proliferation kits (DOJINDO, Japan) according to the manufacturer's instructions. Granular cells were seeded in a 96-well plate at 3,000 cells/well and cultured at 37°C under 5% CO<sub>2</sub> for 0 h, 24 h, 48 h, 72 h, and 96 h before the addition of 10 µL of cell counting kit-8. After incubation for a further 2 h, the optical density value was measured at 450 nm on a microplate reader (Infinite F50, Switzerland). We prepared 6 replicates for each treatment, and the cell growth curve was plotted against time as the x-axis and optical density value against the y-axis.

### **Annexin V-FITC Staining**

Apoptosis was measured using an annexin V-FITC apoptosis kit (DOJINDO) according to the manufacturer's instructions. Granular cells were seeded in 24-well cell culture plates at  $1.5 \times 10^4$  cells/well and cultured for 96 h at 37°C under 5% CO<sub>2</sub>. Subsequently, 300 µL of  $1 \times$  binding buffer and 5 µL of annexin V-FITC were added, and the cells were incubated for 15 min in the dark. After the addition of 5 µL of PI staining solution and 200 µL of  $1 \times$  binding buffer, cells were analyzed within 5 min by flow cytometry. We prepared 3 replicates for each treatment, and the results were analyzed using FlowJo7.6 (BD Biosciences, Bedford, MA).

### **Statistical Analysis**

Data were analyzed using SPSS 19.0 (IBM Corporation, Armonk, NY). Data were expressed as means ± SD. The laying rate, egg quality, blood index, follicle count, and expression of key mTOR signaling pathway-related genes at the mRNA and protein levels were analyzed in 35-wk and 75-wk laying hens by independent sample *t*-tests. Granulosa cell treatment was analyzed by one-way ANOVA. Significant differences at the 0.05 level due to treatments were separated by Duncan's multiple range tests.  $P < 0.05$  was set as the criterion for statistical significance.

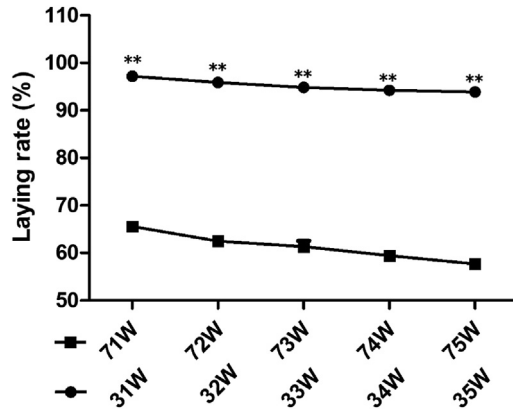
## **RESULTS**

### **The Laying Rate of Hens at Weeks 31 to 35 and Weeks 71 to 75**

As shown in Figure 1, the laying rate declined from week 71 to 75, while the laying rate was maintained at 95% from week 31 to 35. Furthermore, the laying rate from week 71 to 75 was significantly lower than that from week 31 to 35 ( $P < 0.01$ ).

### **The Egg Quality of Hens at Week 35 and Week 75**

As shown in Table 2, the eggshell thickness and strength at week 75 were significantly lower than those



**Figure 1.** The laying rate of hens at week 31 to 35 and week 71 to 75. Data are shown as means  $\pm$  S.D.;  $n = 30$  chicken/group.  $**P < 0.01$  indicates extremely significance compared to the 35-wk hens.

at week 35 ( $P < 0.01$ ), while the egg weight and egg shape index at week 75 were higher than those at week 35 ( $P < 0.01$ ).

### The Concentrations of Antioxidant Enzymes and Immunoglobulin Index in the Plasma of Hens at Week 35 and Week 75

As shown in Table 3, the antioxidant index levels of SOD, GSH-Px, and T-AOC of week 75 were significantly lower than those in week 35 chickens ( $P < 0.01$ ), while the MDA level was significantly higher ( $P < 0.01$ ). The IgM and IgG levels were significantly lower in chickens at week 75 than those in birds at week 35 ( $P < 0.01$ ), while there was no significant difference in IgA levels between chickens at week 35 and week 75.

### The Ovarian Follicle Numbers in Hens at Week 35 and Week 75

As shown in Figure 2, there were fewer follicles in chickens at week 75 than in their week 35 counterparts. The number of LWF, SWF, PE, and MWF of week-75 laying hens were extremely significantly ( $P < 0.01$ ) and significantly ( $P < 0.05$ ) lower than those of week-35 laying hens.

### The Follicular Histology of Hens at Week 35 and Week 75

As shown in Figure 3, the membrane layers of SWF, LWF, SWF, and POF at week 35 were thicker than

**Table 2.** The egg quality of hens at week 35 and week 75.

Item	35 W	75 W	<i>P</i> value
Egg weight (g)	56.25 $\pm$ 2.59 <sup>B</sup>	65.05 $\pm$ 4.01 <sup>A</sup>	0.001
Egg shape index	1.29 $\pm$ 0.04 <sup>B</sup>	1.34 $\pm$ 0.05 <sup>A</sup>	0.001
Eggshell thickness (mm)	0.36 $\pm$ 0.02 <sup>A</sup>	0.32 $\pm$ 0.03 <sup>B</sup>	0.001
Eggshell strength (N)	39.77 $\pm$ 7.04 <sup>A</sup>	34.12 $\pm$ 6.02 <sup>B</sup>	0.001

Values are means  $\pm$  S.D.,  $n = 30$  chicken/group. Means within each row that possess different superscripts differ significantly ( $P < 0.01$ ). If there is no marked letter, the difference is not significant.

**Table 3.** The concentration of antioxidant enzymes and immunoglobulin index in the plasma of hens at week 35 and week 75.

Item	35 W	75 W	<i>P</i> value
MDA (nmol/mL)	4.49 $\pm$ 1.11 <sup>B</sup>	8.03 $\pm$ 1.16 <sup>A</sup>	0.010
SOD (ng/mL)	9.94 $\pm$ 0.89 <sup>A</sup>	6.08 $\pm$ 0.84 <sup>B</sup>	0.002
GSH-Px (ng/mL)	13.25 $\pm$ 2.52 <sup>A</sup>	7.25 $\pm$ 0.25 <sup>B</sup>	0.006
T-AOC (U/mL)	14.75 $\pm$ 2.84 <sup>A</sup>	10.78 $\pm$ 2.40 <sup>B</sup>	0.005
IgA ( $\mu$ g/mL)	207.33 $\pm$ 16.25	197.78 $\pm$ 14.31	0.728
IgG ( $\mu$ g/mL)	2268.98 $\pm$ 140.12 <sup>A</sup>	1765.71 $\pm$ 82.46 <sup>B</sup>	0.001
IgM ( $\mu$ g/mL)	633.20 $\pm$ 37.36 <sup>A</sup>	431.57 $\pm$ 44.99 <sup>B</sup>	0.001

Values are means  $\pm$  S.D.,  $n = 30$  chicken/group. Means within each row that possess different superscripts differ significantly ( $P < 0.01$ ). If there is no marked letter, the difference is not significant.

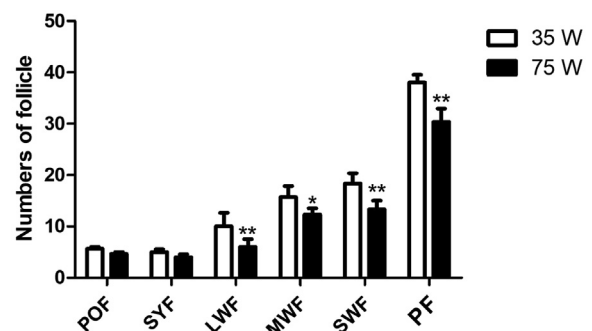
Abbreviations: GSH-Px, glutathion peroxidase; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; MDA, malondialdehyde; SOD, superoxide dismutase; T-AOC, total antioxidant capacity.

those at week 75, the boundary between the intimal and extimal membranes was obvious, and the granular cells were closely arranged.

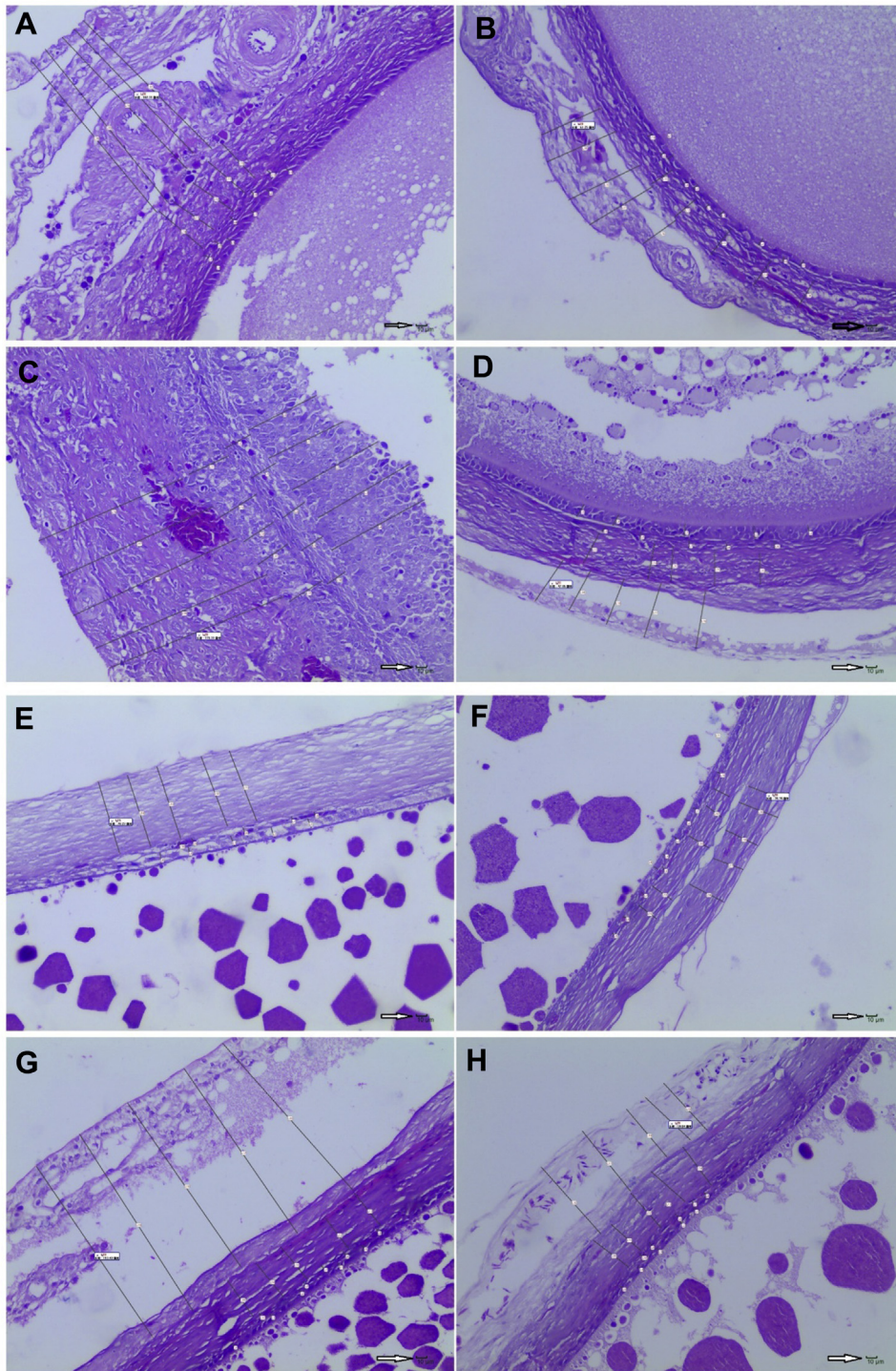
As shown in Table 4, the granular cell layer, intimal membrane layer, extimal membrane layer, and connective layer of the SWF at week 75 were thinner than those at week 35 ( $P < 0.01$ ). The granular cell layer, intimal membrane layer, and connective layer of the LWF at week 75 were lower than those at week 35 ( $P < 0.01$ ). The granular cell layer, extimal membrane layer, and connective layer of the SYF at week 75 were thinner than those at week 35 ( $P < 0.01$ ).

### The mRNA and Protein Expression of mTOR and Downstream Signaling Pathway Components of Hens at Week 35 and Week 75

As shown in Figure 4, significantly lower mTOR mRNA levels were detected in LWF, F3, ovary ( $P < 0.01$ ), and F1 ( $P < 0.05$ ) of week-75 chickens than those of week-35 chickens, while the mRNA expression of SYF was increased significantly ( $P < 0.05$ ). The mTOR protein expression detected



**Figure 2.** The ovarian follicular numbers of hens at week 35 and week 75. POF, preovulatory follicle, diameter  $>10$  mm; SYF: small yellow follicle, diameter is 8 to 10 mm; LWF, large white follicle, diameter is 6 to 8 mm; MWF, medium white follicle, diameter is 4 to 6 mm; SWF, small white follicle, diameter is 2 to 4 mm; PF: primary follicle, diameter  $< 2$  mm. Data are shown as means  $\pm$  S.D.;  $n = 10$  chicken/group.  $*P < 0.05$  indicates significance compared to the 35-wk hens;  $**P < 0.01$  indicates extremely significance compared to the 35-wk hens.



**Figure 3.** The follicle hematoxylin-eosin (HE) staining of hens at week 35 and week 75. (A) 400 $\times$  SWF (small white follicle) of 35-wk birds. (B) 400 $\times$  SWF (small white follicle) of 75-wk birds. (C) 400 $\times$  LWF (large white follicle) of 35-wk birds. (D) 400 $\times$  LWF (large white follicle) of 75-wk birds. (E) 400 $\times$  SYF (small yellow follicle) of 35-wk birds. (F) 400 $\times$  SYF (small yellow follicle) of 75-wk bird. (G) 400 $\times$  POF (preovulatory follicle) of 35-wk birds. (H) 400 $\times$  POF (preovulatory follicle) of 75-wk birds. The scale barns is 10  $\mu$ m.

by Western blot analysis was consistent with the mRNA expression levels.

As shown in [Figure 5](#), compared with week-35 laying hens, the mRNA expression levels of CLIP in SWF, LWF, F3, and ovary were significantly decreased in week-75 laying hens ( $P < 0.05$ ), while significantly increased expression levels were detected in SYF ( $P < 0.01$ ) and F1 follicles ( $P < 0.05$ ). Compared with week-35 laying hens,

we found significantly lower GRB10 mRNA expression in SWF, F1 ( $P < 0.05$ ), LWF, F3, and ovary ( $P < 0.01$ ) in week-75 laying hens, while GRB10 mRNA expression was significantly higher in SYF of week-75 laying hens ( $P < 0.05$ ). Compared with week-35 laying hens, LIPIN1 mRNA expression levels were significantly decreased in LWF, F3, ovary ( $P < 0.01$ ), and SWF ( $P < 0.05$ ) of week-75 laying hens, while the expression level of SYF

**Table 4.** The follicular histology of hens at week 35 and week 75.

Follicle	Item	35 W	75 W	<i>P</i> value
SWF	Granular layer thickness (μm)	9.33 ± 2.12 <sup>A</sup>	5.66 ± 1.01 <sup>B</sup>	0.001
	Intima layer thickness (μm)	7.78 ± 1.79 <sup>A</sup>	4.71 ± 0.83 <sup>B</sup>	0.001
	Extima layer thickness (μm)	48.57 ± 3.47 <sup>A</sup>	22.97 ± 5.66 <sup>B</sup>	0.001
	Connective layer thickness (μm)	158.14 ± 18.31 <sup>A</sup>	72.25 ± 15.35 <sup>B</sup>	0.001
LWF	Granular layer thickness (μm)	104.73 ± 19.88 <sup>A</sup>	12.46 ± 1.50 <sup>B</sup>	0.001
	Intima layer thickness (μm)	19.45 ± 6.34 <sup>A</sup>	5.83 ± 1.10 <sup>B</sup>	0.001
	Extima layer thickness (μm)	32.52 ± 5.04	33.36 ± 3.93	0.684
	Connective layer thickness (μm)	160.94 ± 9.87 <sup>A</sup>	64.88 ± 17.84 <sup>B</sup>	0.001
SYF	Granular layer thickness (μm)	8.13 ± 1.71 <sup>A</sup>	4.72 ± 0.74 <sup>B</sup>	0.001
	Intima layer thickness (μm)	4.77 ± 0.98	4.57 ± 0.58	0.572
	Extima layer thickness (μm)	68.10 ± 2.19 <sup>A</sup>	20.99 ± 2.58 <sup>B</sup>	0.001
	Connective layer thickness (μm)	51.80 ± 5.47 <sup>A</sup>	35.97 ± 3.84 <sup>B</sup>	0.001
POF	Granular layer thickness (μm)	4.58 ± 0.46	4.53 ± 0.79	0.874
	Intima layer thickness (μm)	5.91 ± 1.21 <sup>a</sup>	4.70 ± 0.89 <sup>b</sup>	0.022
	Extima layer thickness (μm)	38.82 ± 2.83	40.13 ± 2.99	0.331
	Connective layer thickness (μm)	191.60 ± 4.93 <sup>A</sup>	82.71 ± 19.95 <sup>B</sup>	0.001

Values are means ± S.D., *n* = 10 chicken/group. Means within each row that possess different lowercase superscripts differ significantly ( $P < 0.05$ ). Means within each row that possess different uppercase superscripts differ significantly ( $P < 0.01$ ). If there is no marked letter, the difference is not significant.

Abbreviations: LWF, large white follicle, diameter 6 to 8 mm; POF, preovulatory follicle, diameter >10 mm; SWF, small white follicle, diameter 2 to 4 mm; SYF, small yellow follicle, diameter 8 to 10 mm.

was significantly increased ( $P < 0.01$ ). Compared with week-35 laying hens, ULK1 mRNA expression in SWF, LWF, F3, F1, and ovary was significantly increased in week-75 laying hens ( $P < 0.01$ ), while levels in SYF were significantly decreased ( $P < 0.05$ ). The 4E-BP1 mRNA levels in SWF, LWF, F3, F1, and ovary of week-75 laying hens were significantly lower than those in week-35 laying hens ( $P < 0.01$ ), while 4E-BP1 mRNA levels in SYF were significantly higher ( $P < 0.01$ ). Western blot analysis showed that 4E-BP1 protein expression levels were consistent with the mRNA expression levels. In all the tissues tested, the S6K mRNA expression levels of week-75 laying hens were significantly lower (LWF, SYF, F3,  $P < 0.01$ ; SWF, F1, ovary,  $P < 0.05$ ) than those in week-35 laying hens.

As shown in Figure 6, compared with week-35 laying hens, we found that RHO mRNA levels were significantly lower in LWF, F3, F1, ovary ( $P < 0.01$ ), and SWF ( $P < 0.05$ ) in week-75 laying hens; however, RHO mRNA levels in SYF were significantly higher ( $P < 0.01$ ). PKC mRNA levels in SWF, LWF, F3, F1, and ovary in week-75 laying hens were significantly lower than those in week-35 laying hens ( $P < 0.01$ ). Western blot analysis showed that the PKC protein expression levels were consistent with the mRNA expression levels. Compared with week-35 laying hens, SGK1 mRNA levels were significantly decreased in LWF, F3 ( $P < 0.01$ ), and ovaries ( $<0.05$ ) in week-75 laying hens, while the expression levels in SYF was extremely significantly increased ( $P < 0.01$ ).

### **Effects of the mTOR-Specific Agonist MHY1485 on the Proliferation of Chicken Granulosa Cells**

As shown in Figure 7, we found that the cell proliferation in the MHY1485-treatment group increased in a

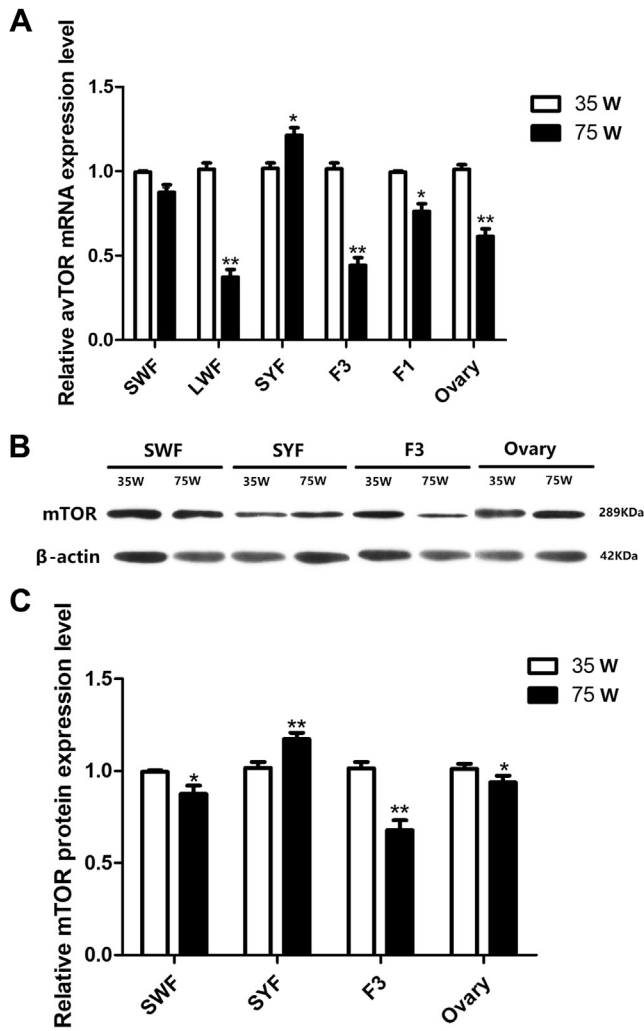
time- and dose-dependent manner, indicating that MHY1485 significantly enhanced the proliferation of chicken granulosa cells.

### **Effects of the mTOR-Specific Agonist MHY1485 on the Apoptosis of Chicken Granulosa Cells**

Apoptosis of chicken granulosa cells was detected using the TUNEL method. As shown in Figures 8A–D, the number of viable cells gradually increased (Q4) and the number of apoptotic cells decreased (Q2, Q3) after 96 h of treatment with MHY1485 in a concentration-dependent manner. Figures 8E, 8F show that compared with the control group, treatment with MHY1485 at 100 μmol/L significantly elevated cell viability ( $P < 0.05$ ), and treatment at 100 μmol/L significantly reduced the apoptosis rate ( $P < 0.05$ ).

### **Effects of mTOR Activation With the Specific Agonist MHY1485 on mTOR Signaling Pathway-Related Genes and Proteins**

As shown in Figure 9A, avTOR mRNA levels were significantly higher in the groups treated with MHY1485 at 10 μmol/L and 100 μmol/L than the levels detected in the control groups ( $P < 0.01$ ). Compared with the control group, treatment with MHY1485 extremely significantly increased the mRNA expression of S6K ( $P < 0.01$ ), and the effects at 10 μmol/L and 100 μmol/L MHY1485 were significantly higher than the effect at 1 μmol/L ( $P < 0.01$ ). Compared with the control group, significantly higher levels of 4E-BP1 mRNA were detected in the groups treated with MHY1485 at 10 μmol/L ( $P < 0.01$ ), and the effect at 10 μmol/L MHY1485 was significantly greater than



**Figure 4.** The mTOR mRNA and protein expression of hens at week 35 and week 75. (A) The mRNA expression of mTOR of hens at week 35 and week 75. (B and C) The mTOR protein expression of hens at week 35 and week 75. Data are shown as means  $\pm$  S.D.;  $n = 10$  chicken/group. \* $P < 0.05$  indicates significance compared to the 35-wk hens; \*\* $P < 0.01$  indicates extremely significance compared to the 35-wk hens.

the effect at  $1 \mu\text{mol/L}$  and  $100 \mu\text{mol/L}$  ( $P < 0.05$ ). PKC mRNA expression in the MHY1485 treatment group was significantly higher than that in the control group ( $P < 0.01$ ). As shown in Figures 9B–F, the protein expression results were consistent with the mRNA expression results. The expression levels of mTOR, S6K, p-mTOR, and p-S6K proteins increased with increasing MHY1485 concentration.

## DISCUSSION

In the present study, we compared the laying rate, follicle number, antioxidant index, and immune index of laying hens at 35 and 75 wk of age and explored the expression of key genes and proteins of the TOR pathway at the different ages.

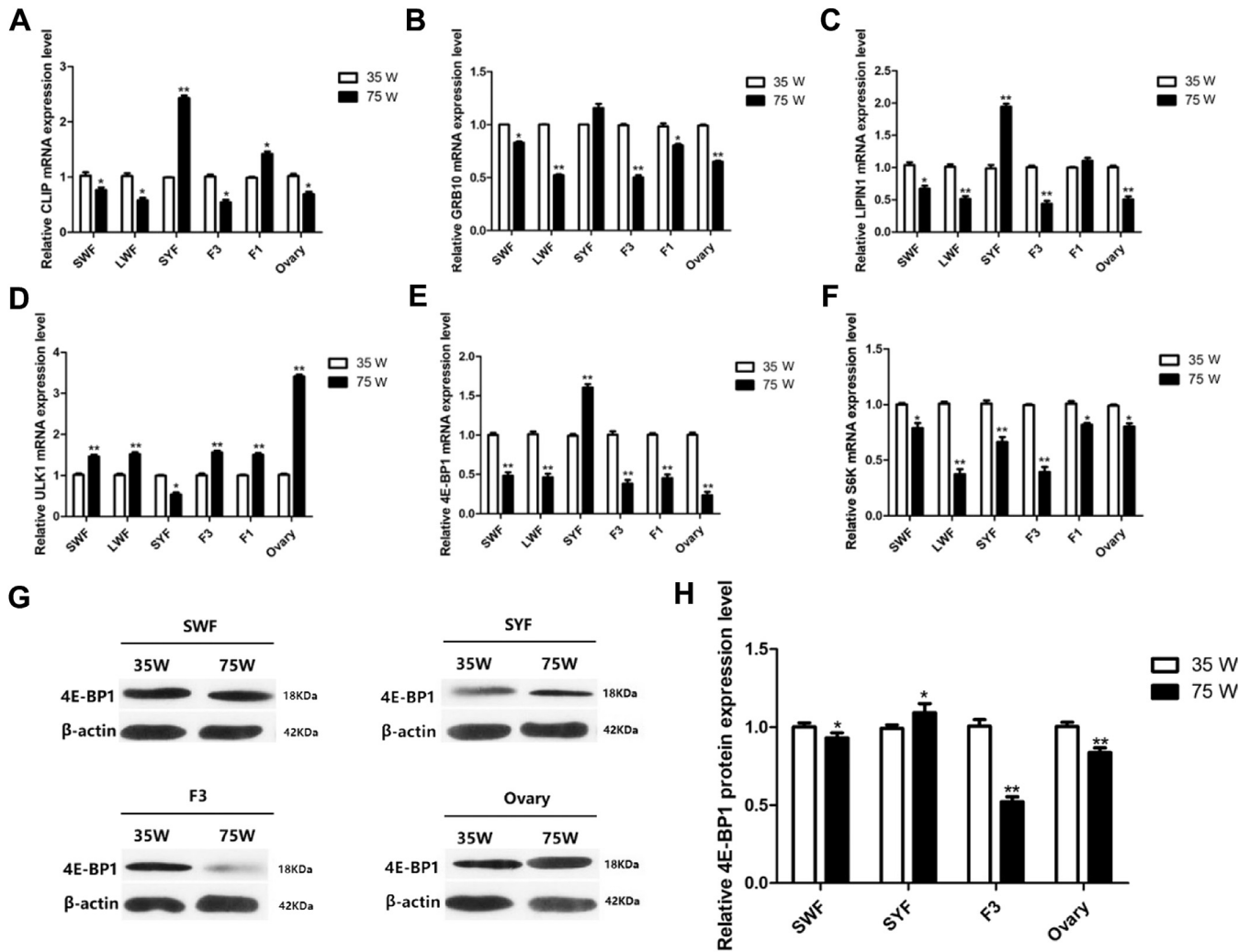
Aging is a normal and complex biological process that is common to all living organisms. As they age, the physiological functions of cells and tissues decline. Aging of laying hens is reflected mainly by a decrease in egg

production rate, eggshell thinning, an increase in the average egg laying interval, decreasing follicle number, and an increasing follicular atresia rate. The main reason for the decline in laying rate is ovarian aging and hypo-function during late egg production. Liu et al. reported that the egg production rate of laying hens increased with age from 90 to 280 d and was followed by a downward trend from 580 d (Liu et al., 2018). In the present study, we found that the laying rate decreased from 71 to 75 wk, while the laying rate was maintained at 95% in hens aged 31 to 35 wk. Furthermore, the laying rate of hens aged 71 to 75 wk was significantly lower than that of hens aged 31 to 35 wk. The rapid decline in egg production rate with age in laying hens may be related to the suppression of follicular development and may also be related to follicular atresia and abnormal ovulation.

Research has shown that ovarian oxidative stress increases with age and that the expression levels of antioxidation-related genes decreased (Eichenlaubritter et al., 2011; Lim and Luderer, 2011). In our study, we found that the expression of antioxidant index levels (SOD, GSH-Px, and T-AOC) in week-75 hens was significantly lower than that in week-35 hens. The MDA levels in week 75 was also significantly higher than those in their week-35 counterparts. SOD activity is an important indicator of antioxidant capacity (Ragel et al., 2006), and T-AOC levels reflect the T-AOC. SOD and CAT gene expression are downregulated in the follicular granulosa cells in older women (Tatone et al., 2006). Güney et al. (2013) reported that the T-AOC level in the liver and heart was significantly decreased in aging rats (Güney et al., 2013), which is consistent with our findings. MDA is an indicator of lipid peroxidation, and its level can reflect the degree of free radical-induced cell damage. In a random sample of 1,139 women (35–75 yr), Pinchuk et al. (2019) found that total glutathione (GSH) decreased with age, while MDA increased, with significant changes occurring at approximately  $53 \pm 2$  yr of age (Pinchuk et al., 2019). Thus, our findings provide further evidence that the decrease in endogenous antioxidant activity reflects the reduced antioxidant capacity of the aging hens.

Follicle number, which is a key factor of laying performance, is related to breed (Johnson et al., 2009), age (Lillpers and Wilhelmson, 1993), and nutrition (Mcderment et al., 2012). The number of follicles determines the laying rate of hens, with low follicle numbers associated with a low laying rate. As the age of laying hens increased, the number of follicles at different developmental stages decreased, while the number of atretic follicles increased. In the present study, we found fewer follicles in week-75 hens than in week-35 hens. The numbers of LWF, SWF, PE, and MWF of week-75 laying hens were extremely significantly and significantly lower than those of week-35 laying hens. Researchers have reported large differences in follicle numbers between old laying hens (e.g., aged  $>1$  yr) and laying hens at the peak stage (Ciccione et al., 2003). Comparisons of the number of graded follicles in



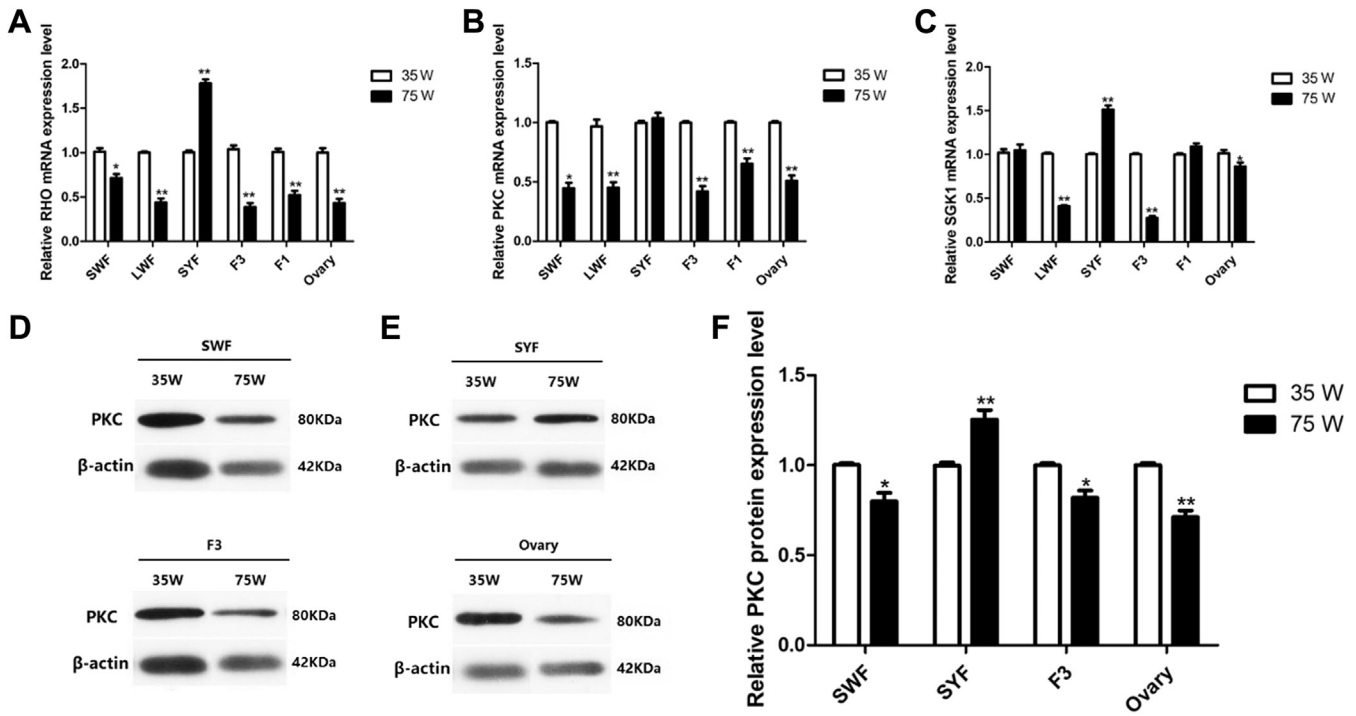


**Figure 5.** The mTORC1 downstream components mRNA and protein expression of hens at week 35 and week 75. (A–F) The mRNA expression of CLIP, GRB10, LIPIN1, ULK1, 4E-BP1, and S6K of hens at week 35 and week 75. (G and H) The 4E-BP1 protein expression of hens at week 35 and week 75. Data are shown as means  $\pm$  S.D.;  $n = 10$  chicken/group. \* $P < 0.05$  indicates significance compared to the 35-wk hens; \*\* $P < 0.01$  indicates extremely significance compared to the 35-wk hens.

the ovary of laying hens at different ages showed that the average number of follicles in the laying hens at week 28 was 7.8 and decreased to 6.3 at week 76 (Zakaria, 1983). In another study, the number of graded follicles in laying hens at week 40 was 4.6 but was reduced to 0.83 by week 160 (Joyner et al., 1987). Studies have shown that the number of follicles in laying hens increased sharply from day 150 to day 280, followed by a significant decrease from day 280 to day 580 (Liu et al., 2018). The proliferation, differentiation, and apoptosis rates of poultry follicle granulosa cells dominate the development of follicles and determine their atresia. HE staining of ovarian tissue sections showed that the thickness of the granular layer, the inner and outer membrane layer, and the connective layer of the follicles in aged laying hens was significantly lower than that at the peak. Hens with low laying rates have more follicular atresia and thinner membranes and granular layers (Li et al., 2017). In accordance with these studies, we showed that the number of follicles decreased as the laying

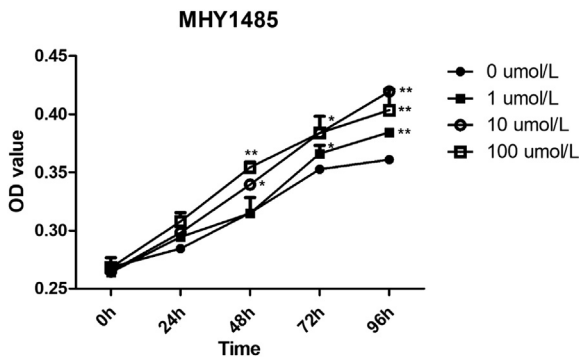
hens aged, which is consistent with the change in the egg laying rate.

The mTOR signaling pathway is important in regulating cell growth and proliferation. mTOR, which consists of rapamycin target protein complex 1 (mTORC1) and rapamycin target protein complex 2 (mTORC2), represents a class of evolutionarily conserved Ser/Thr protein kinases belonging to the phosphoinositide-associated kinase family. mTORC1 regulates cell growth and autophagy, while mTORC2 regulates cytoskeletal dynamics and actin organization (Dowling et al., 2010; Ikenoue et al., 2014). In the present study, we found that the mRNA expression levels of TOR in LWF, F3, F1, and ovary were significantly decreased in week-75 laying hens compared with those in week-35 hens, while the mRNA expression of SYF was increased significantly. In laying hens, follicular development follows a strict hierarchy resulting from a process of orderly growth divided into 2 major systems: pregradation follicles and hierarchical follicles. The POF are F1, F2, F3,



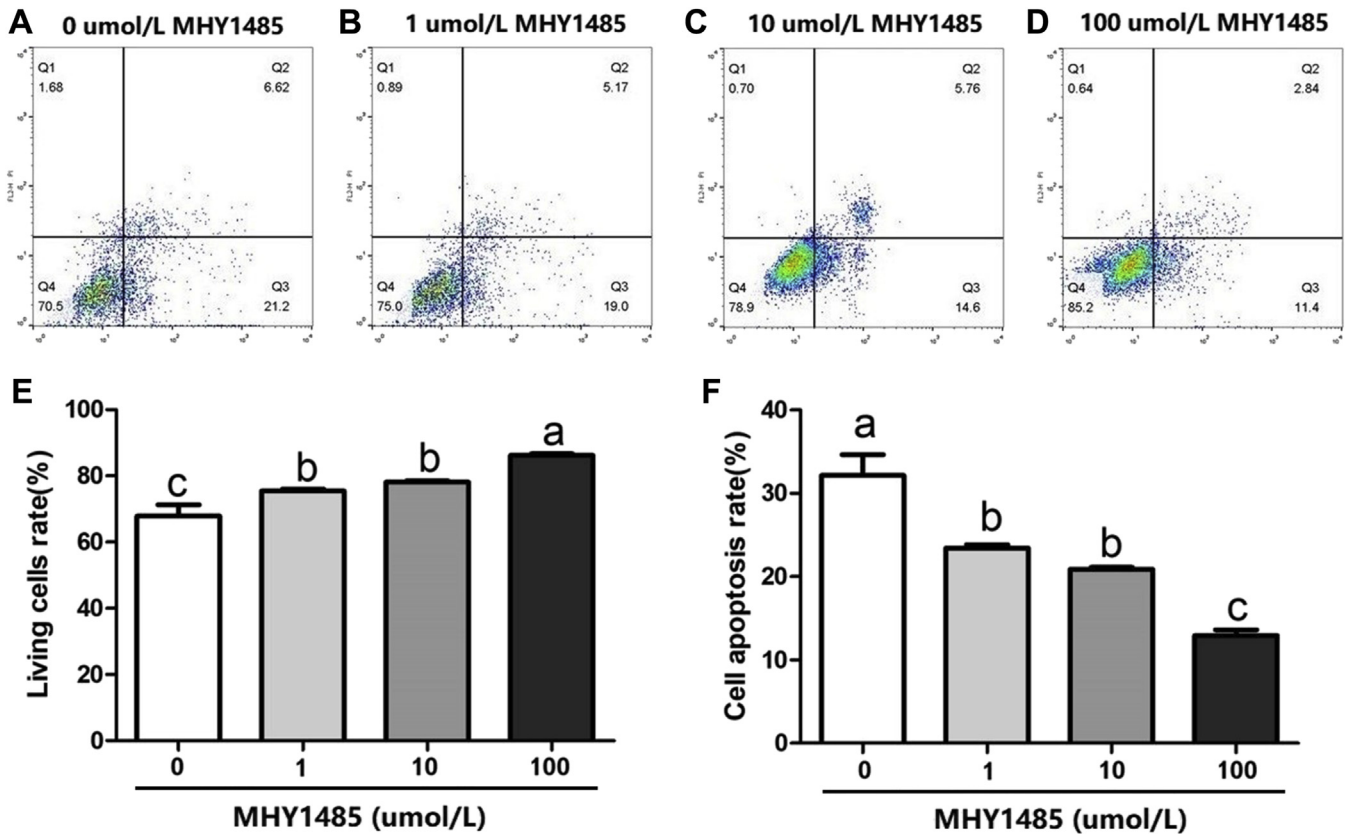
**Figure 6.** The mTORC2 downstream components mRNA and protein expression of hens at week 35 and week 75. (A–C) The mRNA expression of RHO, SGK1 and PKC of hens at week 35 and week 75. (E and F) The PKC protein expression of hens at week 35 and week 75. Data are shown as means ± S.D.; n = 10 chicken/group. \**P* < 0.05 indicates significance compared to the 35-wk hens; \*\**P* < 0.01 indicates extremely significance compared to the 35-wk hens.

F4, F5, and F6 in descending order. The prehierarchical follicles are divided into SYF (8–10 mm in diameter), LWF (6–8 mm), MWF (4–6 mm), SWF (2–4 mm), and primary follicles (<2 mm) according to their diameter (Johnson, 2015). After F1 ovulation, a follicle from the SYF pool replaces the previous F6 follicle in a process known as selection (Shen et al., 2017). Generally, only 5% of follicles develop into POF (Gilbert et al., 1983). Thereafter, the follicles are maintained in an orderly preovulatory hierarchy (Johnson, 2015). Therefore, a thorough understanding of the process of follicular development and its mechanisms could aid in significantly improving egg production.



**Figure 7.** The effect of different concentrations of MHY1485 (an mTOR agonist) on the proliferation of chicken granule cells (n = 6). Data are shown as means ± S.D.; \**P* < 0.05 indicates significance from respective control values; \*\**P* < 0.01 indicates extremely significance from respective control values.

In the present study, we found that the mRNA levels of the mTORC1 downstream genes *CLIP*, *GRB10*, *LIPIN1*, *4E-BP1*, *S6K*, *RHO*, and *SGK* in SWF, LWF, F1, F3, and ovaries of week-75 laying hens were significantly lower than those of week-35 laying hens, while the SYF mRNA level was significantly higher. Growth factor receptor binding protein 10 (GRB10) plays an important role in regulating cell growth and metabolism, embryo growth and glucose metabolism, and fat storage (Langlais et al., 2004). As a downstream gene of mTORC1, GRB10 negatively regulates the insulin signaling pathway (Yu et al., 2011). Overexpression of Grb10 can affect Ins-regulated blood glucose transport (Yamasaki-Ishizaki et al., 2007), reduce DNA synthesis, and inhibit cell division and proliferation (Shiura et al., 2005). LIPIN (Lipin1) is an important regulator of lipid synthesis and is highly expressed in adipose tissue (Huffman et al., 2002). Lipin1 plays an important role in adipogenesis and adipocyte development (Koh et al., 2008). Poultry reproduction performance is closely related to fat deposition and metabolism (Walzem and Chen, 2014). In the late laying period, the laying rate of the laying hens decreases continuously with age, and the energy output is low. At this time, the excess energy absorbed by the hen from the diet is deposited in the form of fat, particularly in the abdominal area. The most deposits further affect the decline in egg production (Goraga et al., 2010; Zhang et al., 2018). As 2 important effectors downstream of mTORC1, phosphorylation of eukaryotic initiation factor 4E binding protein-1 (4E-BP1) and ribosomal protein 6kinase (S6K), regulates

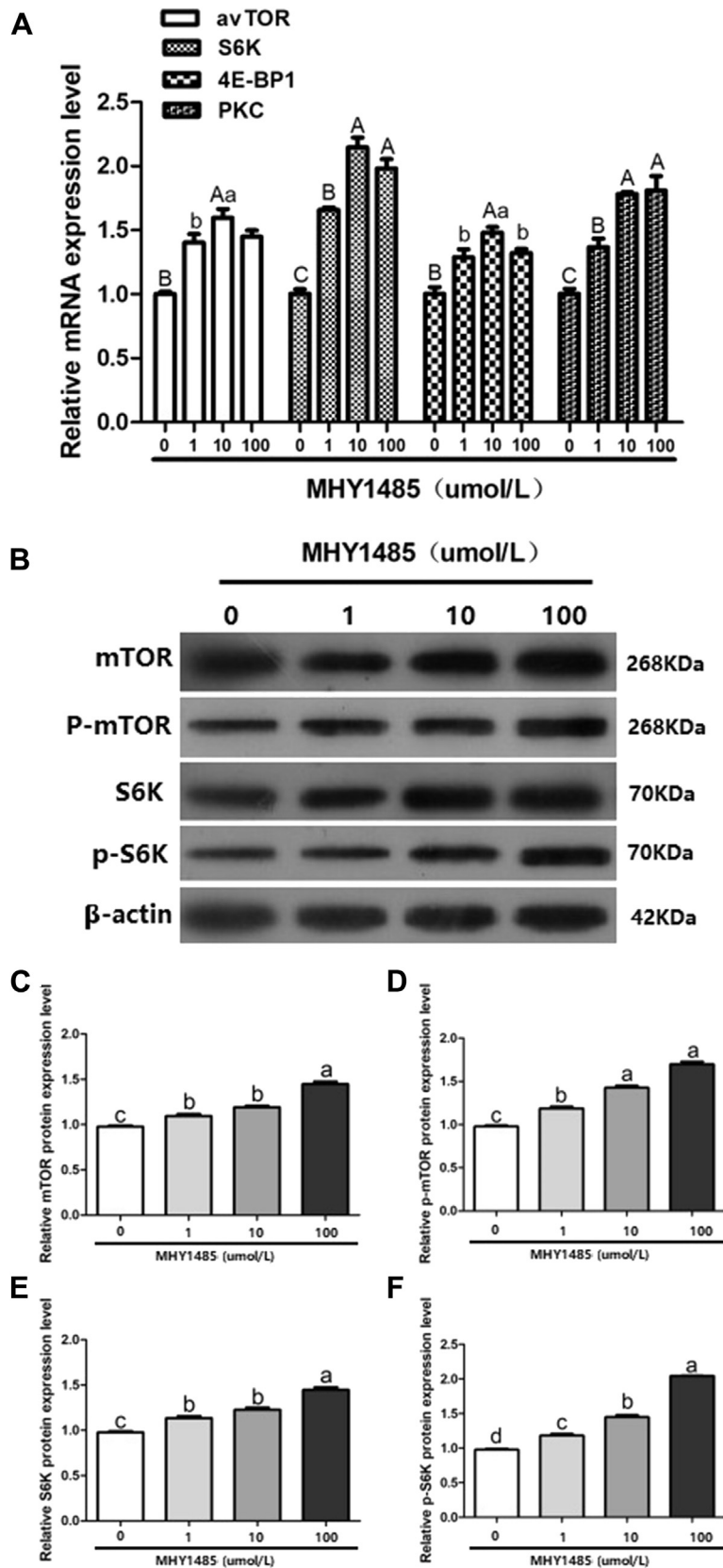


**Figure 8.** The effect of different concentrations of MHY1485 (an mTOR agonist) on the apoptosis of chicken granule cells. (A) Control group; (B) 1  $\mu\text{mol/L}$  MHY1485; (C) 10  $\mu\text{mol/L}$  MHY1485; (D) 100  $\mu\text{mol/L}$  MHY1485; (E) granular cells living rate; (F) granular cells apoptosis rate ( $n = 3$ ). Data are shown as means  $\pm$  S.D.; A and B,  $P < 0.05$  indicates significance from respective control values.

the initiation of mRNA translation and promotes cell growth and development (Corradetti and Guan, 2006; Saxton and Sabatini, 2017). Research has shown that S6K1 and 4E-BP1 mRNA expression decreases in 14-day-old chicks compared with that in 3-day-old chicks (Deng et al., 2014). Phosphorylation of eIF4E decreases with age (Kimball et al., 2004), which is consistent with our findings. As a downstream gene of mTORC2, PKC is crucially involved in cell proliferation, differentiation, and apoptosis. PKC mRNA expression levels in SWF, LWF, F3, F1, and ovary of week-75 laying hens were significantly lower than those in week-35 laying hens ( $P < 0.01$ ). mTORC2 is responsible for the phosphorylation of PKC and posttranslational processing (Ikenoue et al., 2014). A PKC agonist was reported to activate rat spinal cord glial cells, while an inhibitor reduced cell activation, indicating that decreased PKC expression inhibits cell growth. ULK is an important promoter of autophagy and is directly phosphorylated by mTOR to negatively regulate autophagy, thereby affecting cell growth (Jung et al., 2010). In this study, ULK1 mRNA expression in SWF, LWF, F3, F1, and ovary was significantly increased in week-75 laying hens compared with that in week-35 laying hens, while SYF decreased significantly. Autophagy of the follicles of the laying hens is increased in the late laying period, with increased granulocyte apoptosis due to the increase in autophagy. Follicle atresia also increased, leading to a

reduction in the laying rate in the late laying period. Western blot analysis confirmed that expression of mTOR, 4E-BP1, and PKC at the protein level was consistent with the expression levels of mRNA. In our study, we found that SYF follicles exhibited a different mTOR expression pattern in aging layers compared with the pattern of expression in other follicles. The differences in egg production traits of chickens are determined mainly by the regulation of primordial follicle recruitment initiation, pregrade follicular development, dominant follicular recruitment, follicular hierarchy establishment, and ovulation. Therefore, the development of SYF follicles is particularly important, and a thorough understanding of the process of follicular development and its mechanisms could aid in significantly improving egg production.

To further verify the relationship between mTOR signaling pathway and egg production rate, we treated chicken granulosa cells with the mTOR agonist MHY1485 to activate the mTOR pathway. Our results showed that the proliferation of chicken granulosa cells was enhanced, and apoptosis was significantly inhibited, thus demonstrating the close relationship of the mTOR pathway with the regulation of chicken granulosa cell proliferation and growth. This process was accompanied by a significant upregulation of the mTOR signaling pathway marker molecule mTOR and important downstream genes *S6K*, *4E-BP1*, and



**Figure 9.** The effect of MHY1485 (an mTOR agonist) activated the mTOR on mTOR pathway-related genes and protein expression. (A) The avTOR, S6K, 4E-BP1, and PKC mRNA expression of MHY1485 treatment of the granulosa cells. (B-F) The mTOR, p-mTOR, S6K, and p-S6K protein expression of MHY1485 treatment of the granulosa cells (n = 6). Data are shown as means ± S.D.; A-C, *P* < 0.05 indicates significance from respective control values, A-C, *P* < 0.01 indicates extremely significance from respective control values.

*PKC*. The expression of mTOR, p-mTOR and S6K, and p-S6K proteins were also upregulated, indicating that the mTOR signaling pathway influences chicken granulosa cell biology and function. Furthermore, activation of the mTOR signaling pathway enhances granulosa cell proliferation, which leads to an elevation in the number of developing follicles and an increase in egg production.

## CONCLUSION

In the present study, we observed lower levels of SOD, GSH-Px, and T-AOC, but higher levels of MDA, as well as downregulated expression of key mTOR signaling pathway-related genes at the mRNA and protein levels in 75-week-old laying hens compared with their 35-week-old counterparts. The mTOR agonist MHY1485 significantly increased granulosa cell proliferation and inhibited cell apoptosis. Enhancement of the expression of genes and proteins of downstream of the mTOR pathway indicates that the growth and development of chicken granulosa cells may be regulated by the mTOR pathway. This study indicates that follicular development is suppressed by a decline in ovarian function. Moreover, the level of oxidative stress in the laying hens is increased, leading to a rapid decline in laying rate. The proportion of laying hens in the late laying period is relatively large; therefore, extending the utilization period of the laying hens in the late laying period is of great significance. Although age-related ovarian failure in laying hens cannot be reversed, recent strategies have focused on improving reproductive performance with age. Proximal factors and processes that mediate follicle selection can either extend or decrease the length of the laying sequence and thus directly influence overall egg production. The results of this study further our understanding of the key role of mTOR in ovarian aging in laying hens and highlight strategies for delaying ovarian aging by targeting the mTOR signaling pathway.

## ACKNOWLEDGEMENTS

This research was supported by the China Agriculture Research System (CARS-40-K20), Hebei Natural Science Foundation (C2020204163), and National Natural Science Foundation (32002220).

## DISCLOSURES

The authors declare no conflicts of interest.

## SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2020.10.005>.

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