

Melatonin regulates chicken granulosa cell proliferation and apoptosis by activating the mTOR signaling pathway via its receptors

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ABSTRACT Melatonin is a key regulator of follicle granular cell maturation and ovulation. The mammalian target of rapamycin (mTOR) pathway plays an important role in cell growth regulation. Therefore, our aim was to investigate whether the mTOR signaling pathway is involved in the regulation of melatonin-mediated proliferation and apoptotic mechanisms in granulosa cells. Chicken follicle granular cells were cultured with melatonin (0, 2, 20, or 200 $\mu\text{mol/L}$) for 48 h. The results showed that melatonin treatment enhanced proliferation and suppressed apoptosis in granular cells at 20 $\mu\text{mol/L}$ and 200 $\mu\text{mol/L}$ ($P < 0.05$) by upregulation of cyclin D1 ($P < 0.01$) and Bcl-2 ($P < 0.01$) and downregulation of P21, caspase-3, Beclin1, and LC3-II ($P < 0.01$). The effects resulted in the activation of the mTOR signaling pathway by increasing the expression of avTOR, PKC, 4E-BP1, S6K ($P < 0.05$), p-mTOR, and p-S6K. We added an mTOR activator and inhibitor to the cells and identified the optimal dose (10 $\mu\text{mol/L}$ MHY1485 and 100 nmol/L rapamycin) for subsequent experiments. The combination

of 20 $\mu\text{mol/L}$ melatonin and 10 $\mu\text{mol/L}$ MHY1485 significantly enhanced granulosa cell proliferation ($P < 0.05$), while 100 nmol/L rapamycin significantly inhibited proliferation and enhanced apoptosis ($P < 0.05$), but this action was reversed in the 20- $\mu\text{mol/L}$ melatonin and 100-nmol/L rapamycin cotreatment groups ($P < 0.05$). This was confirmed by mRNA and protein expression that was associated with proliferation, apoptosis, and autophagy ($P < 0.05$). The combination of 20 $\mu\text{mol/L}$ melatonin and 10 $\mu\text{mol/L}$ MHY1485 also activated the mTOR pathway upstream genes PI3K, AKT1, and AKT2 and downstream genes PKC, 4E-BP1, and S6K ($P < 0.05$), as well as protein expression of p-mTOR and p-S6K. Rapamycin significantly inhibited the mTOR pathway-related genes mRNA levels ($P < 0.05$). In addition, activation of the mTOR pathway increased melatonin receptor mRNA levels ($P < 0.05$). In conclusion, these findings demonstrate that melatonin regulates chicken granulosa cell proliferation and apoptosis by activating the mTOR signaling pathway via its receptor.

Key words: melatonin, chicken granulosa cell, mTOR signaling pathway, proliferation, apoptosis

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INTRODUCTION

The follicle is the basic functional unit of the chicken ovary. Although there are approximately 480,000 follicles in newborn hens, it has been found on screening the ovaries of adult hens that only hundreds of follicles have the opportunity to ovulate (Onagbesan, 2009).

The growth and development of follicles is a complex process with a strictly ordered hierarchy. Avian follicular development has certain unique features in that the proportion of primordial follicles that develop into the small yellow follicle (diameter < 8 mm) pool accounts for only 5% of the total number of follicles, and only one dominant follicle is selected to progress to ovulation (Johnson and Woods., 2009), while the rest of the follicles undergo atresia. Poultry follicular development and the follicular selection process determine the number of mature follicles, so these are key factors affecting poultry reproduction ability. It is unclear how follicular selection occurs, but studies have shown that granulosa cells play an important role in follicular selection throughout the follicular development process

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(Johnson, 2015). Research has confirmed that follicular selection and atresia are the results of apoptosis of granulosa cells (Nakayama, 2000; Manabe, 2004). Granulosa cell apoptosis leads to a decrease in the number of follicles capable of further development and is the main cause of the decline in egg production rate in poultry. Thus, reducing granulosa cell apoptosis and atresia is an important means to increase the egg production rate in poultry.

Melatonin (N-acetyl-5-methoxytryptamine; MW = 232) was first discovered in the pineal gland and is important in the regulation of circadian rhythms (Weissová, 2018). Melatonin plays an important regulatory role in many physiological systems including female reproduction (Wang et al., 2014) and innate immunity (Zhou, 2016). Melatonin also has antiradiation (Fernández-Gil, 2017), anticancer (Söderquist, 2016), antiaging (Tamura et al., 2017), and antioxidation (Reiter, 1993; Mehaisen, 2015) properties and can scavenge reactive oxygen species (Zhang et al., 2006). Studies have found that melatonin levels in the blood decrease with increasing age of the laying hens, and exogenous melatonin can significantly improve the rate of egg laying in aged laying hens (Jia, 2016). Previous studies have reported that melatonin-binding sites were present in the ovaries (Ayre, 1992; Sundaresan, 2009) and granulosa cells (Murayama, 1997) of chickens, which indicates that melatonin acts directly on the ovary and the granulosa cells (Kang et al., 2009; Ahmad, 2012) to regulate its function (Fiammetta, 2010). Melatonin also regulates cell proliferation and apoptosis depending on the cell type (Sainz, 2003). It stimulates proliferation, differentiation, and maturation; induces apoptosis; and enhances autophagy in normal cells, such as bovine granulosa cells (Ahmad, 2012), rat ovarian follicles (Maganhin, 2013), and human granulosa cells (Taniguchi, 2009). In contrast, melatonin can inhibit proliferation and promote apoptosis in tumor cells (Wang et al., 2012; Liu, 2013). The protective effect of melatonin on cells may be due to its scavenging of reactive oxygen species and stimulating the activity of antioxidant enzymes (Tamura et al., 2013; Tan, 2015) or it may be regulated by melatonin receptors MTR1 and MTR2 to activate other signaling pathways (Zhang et al., 2019).

Another factor that may be involved in the follicular maturation of granulosa cells is the mTOR signaling pathway. mTOR Plays a pivotal role in the integration of cellular signals arising from mitogens, stress, and nutrition and also has effects on regulating cell growth and proliferation (Cheng, 2015). The signaling pathway of mTOR can integrate growth factors and nutrients in many cells, so it may be the basis of follicle maturation in granulosa cells (Meredith, 2000). Currently, researchers have found that mTOR signaling pathways regulate ovarian function and granulosa cell development and ovulation (Dowling, 2009). The mTOR signaling pathway is also involved in the process by which melatonin regulates the proliferation and development of mouse follicular cells (Behram, 2017). The specific regulatory mechanism of mTOR on chicken granulosa cells is still not clear. Whether the mTOR pathway is also involved in melatonin-mediated

proliferation and apoptosis of granulosa cells in poultry is also unknown.

Melatonin has been shown to be involved in the growth and proliferation of follicles, but the mechanism of action of melatonin on follicular development is still unclear and requires further clarification. The mechanisms by which melatonin regulates cell proliferation, apoptosis, and autophagy and whether the mTOR signaling pathway is involved in these processes still need to be elucidated. The present study investigated the effects of melatonin on cell proliferation, apoptosis, and autophagy of chicken granulosa cells and whether these effects are mediated by activation of the mTOR signaling pathway. Moreover, MHY1485 (an mTOR agonist) and rapamycin (an mTOR inhibitor) were used to further confirm the role of the mTOR signaling pathway in melatonin-mediated regulation of chicken granulosa cell proliferation, apoptosis, and autophagy.

MATERIALS AND METHODS

The Purpose and Significance of the Study

To study the effects of melatonin on chicken granulosa cell proliferation, apoptosis, and autophagy, cells were treated with different concentrations of melatonin for 48 h. To verify whether melatonin can activate the mTOR signaling pathway, the mRNA levels of mTOR downstream genes, including mTOR, PKC, 4E-BP1, S6K, phosphorylated mTOR, and phosphorylated S6K were analyzed by quantitative real-time PCR (qRT-PCR) and Western blot. To further confirm whether the mTOR pathway is involved in the regulation of melatonin-mediated proliferation, apoptosis, and autophagy of chicken granulosa cells, an mTOR activator and an mTOR inhibitor were added to the cells or in combination with melatonin in the culture medium. After 48 h of treatment, the cells were harvested and analyzed by cell counting kit-8 (CCK-8), the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method, qRT-PCR, and Western blot.

Animals and Granulosa Cell Collection

Healthy Dawu jinfen laying hens aged 245 D that were laying in regular sequences were used in these studies. The laying hens were kept in an environment with temperature $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$, relative humidity $65 \pm 10\%$, and a light:dark cycle of 16:8 h at the Experimental Breeding facility of Hebei Agricultural University (Hebei, China). Water and food were available ad libitum during the entire experimental period. Chickens were euthanized by cervical dislocation within 3 h after laying eggs. The experimental protocol was approved by the Animal Care and Use Committee of the Hebei Agriculture University.

Granulosa Cell Culture and Treatments

Small yellow follicle (6-8 mm in diameter) were removed from the ovary and washed with ice-cold PBS (Gibco, New York). First, the membrane layer was removed and separated from the granular cell layer and washed with PBS. Trypsin-EDTA was added (2 ml, 0.25%) for digestion of the cells (37°C for 2.5 min), then 2 ml of M199 medium (containing 10% fetal bovine serum, 1% penicillin-streptomycin) was added to terminate the digestion. The first granulosa cells were evenly spread in a 6-well plate and placed in an incubator (37°C, 5% CO₂, saturated humidity). After the cells had attached to the plate, 2 mL of M199 medium (Gibco) was added. The culture solution was changed every 3 D. The first generation cells were then treated with M199 medium supplemented with 0, 2, 20, or 200 µmol/L of melatonin (Sigma) or 10, 100, and 1,000-nmol/L rapamycin (an mTOR inhibitor; MCE) or 1-, 10-, and 100-µmol/L MHY1485 (an mTOR agonist; MCE), and incubated for 48 h. The maximal effects were found at 20 µmol/L melatonin, 100 nmol/L rapamycin, and 10 µmol/L MHY1485. In addition, after treatment with 20 µmol/L melatonin for 2 h, 100 nmol/L rapamycin or 10 µmol/L MHY1485 was added, in combination with 20 µmol/L melatonin treatment, for 48 h. Each concentration gradient was repeated 3 times. The effects of the application of melatonin and the mTOR activator and inhibitor are seen in Figure 1.

Cell Proliferation Assay

Cell proliferation was analyzed using the CCK-8 kit (DOJINDO, Japan) according to the manufacturer's instructions. Cultured chicken granulosa cells were diluted at a concentration of 5×10^5 cells/ml and plated in 96-well plates. After culturing for 48 h in the incubator (37°C, 5% CO₂, saturated humidity), 10-µL CCK-8 dye was added before incubating for an additional 2 h. The optical density was determined at a wavelength of 450 nm on a microplate reader. Each treatment had 6 replicates.

Cell Apoptosis Assay

The apoptosis of granulosa cells was detected by using the Annexin V-FITC-PI double-staining assay (DOJINDO, Japan) according to the kit's instructions. Cultured granulosa cells were separated into 24-well plates and washed with precooled PBS. Then 300 µL of cells suspended in 1× binding buffer were added, followed by 5 µL of Annexin V-FITC. The plates were then sealed and incubated in the dark at room temperature for 15 min. Then



Figure 1. The time points of the application of the melatonin and the mammalian target of rapamycin (mTOR) activator and inhibitor.

5 µL of propidium iodide and 200 µL of 1× binding buffer were added 5 min before flow cytometry was performed. The results were analyzed using FlowJo7.6 (BD Biosciences, Bedford, MA) to analyze cell dispersion points. Each treatment had 3 replicates.

qRT-PCR Analysis

Total RNA from chicken granulosa cell samples (from 3 replications) was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). The quantity and purity of the total RNA were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The RNAs were reverse transcribed by using the SuperScript III First-Strand Synthesis Super-Mix for qRT-PCR (Invitrogen, Carlsbad, CA). The synthesized cDNA was amplified using a 20-µl PCR reaction system containing 1 µl of cDNA, 10 µl Power SYBR Green PCR Master Mix (Roche, Basel, Switzerland), 0.5 µl PCR forward primer, 0.5 µl PCR reverse primer (Huada, Beijing, China), and 8 µl ddH₂O. The sequences of primers for the qPCR are listed in Table 1. All samples were assayed in triplicate. All data were analyzed using the $2^{-\Delta\Delta C_t}$ method (Livak, 2001).

Western Blot Analysis

Total protein from chicken granulosa cells was extracted using the total extraction kit (Sigma). Then, 20 µl of the protein extracts were separated on 12% SDS polyacrylamide gels. After electrophoresis, proteins were electroblotted onto polyvinylidene fluoride membranes (Millipore, MA) at 100 V for 60 min. Tris-buffered saline (TBS) containing 5% nonfat milk was added for 1 h at room temperature, followed by overnight incubation at 4°C with primary antibodies. Membranes were washed 3 times with TBS-T for 20 min to remove unbound primary antibodies, then incubated for 1 h at 37°C with secondary antibodies conjugated to horseradish peroxidase and washed 5 times with TBS-T for 5 min. Finally, signals were detected by chemiluminescent ECL detecting reagents (GE) and scanned using the Super Signal Extended Duration Substrate (Thermo Fisher Scientific, Waltham, MA). The same procedures were repeated for analysis of with primary antibodies against Cyclin D1 (Abcam; 1:10,000 dilution), LC3-II (Abcam; 1:800 dilution), caspase-3 (Abcam; 1:500 dilution), mTOR (Abcam; 1:2000 dilution), P-mTOR (Abcam; 1:1500 dilution), S6K (Abcam; 1:1000 dilution), and p-S6K (Abcam; 1:1000 dilution) and secondary antibodies goat antirabbit IgG (Abcam; 1:5000 dilution) or goat antimouse IgG (Abcam; 1:5000 dilution). An antibody against β-actin (Abcam; 1:1000 dilution) was used as an internal control. The BandScan 5.0 image software was used for densitometric analysis.

Data Analysis

Data were recorded and analyzed using SPSS 19.0 (IBM Corporation). All data were analyzed by one-

Table 1. Primers for qRT-PCR.

Gene	Genbank accession	Primer sequences (5' to 3')	Size (bp)
<i>MTR1</i>	NM_205362.1	F CACCATCCTCATCTTCACCATC R TTGCCAGGCTTACCACAAATA	119
<i>MTR2</i>	NM_001293103.1	F CTGTTGTGAGCTTCTGCTATCT R CTTGGTTTCAGTCTTGGCTTTG	94
<i>Cyclin D1</i>	NM_205381.1	F GCACAGCAGCACAAACGTATC R ATCTCGCACATCAGTGGGTG	84
<i>P21</i>	NM_204396.1	F AGCAGATCCAGAACGACTTTG R GGACCCTCTCCCCTTGA	123
<i>Bcl-2</i>	NM_205339.2	F TTGTACGGCAACAGTATGAGG R TCCAAGATAAGCGCCAAGAG	111
<i>Caspase-3</i>	NM_204725.1	F GGCTGTAGAGGAACAGAATTAG R AGTAGCCTGGAGCAGTAGAA	125
<i>Lc3-II</i>	XM_417327.6	F AATCCACCCAGGCTTTC R TCCTTCTCCTGCTCGTAGAT	92
<i>Beclin1</i>	NM_001006332.1	F TGGAGAACCAGATGCGTTATG R ACGGCCAAGTCTGAAGTTATT	128
PI3K	NM001004410	F CGGATGTTGCCTTACGGTTGT R GTTCTTGTCTTGAGCCACTGAT	162
AKT1	AF039943	F TGATGGCACATTTCATTGGCTAC R TGTGGTTTGGTTAGGTCGTTCTGTCT	122
AKT2	AF181260	F CCGAAGTGCTGGAGGACAAC R CGCTCGTGGTCCTGGTTGTA	115
<i>4E-BP1</i>	XM_424384.6	F GCGAATGTAGGTGAAGAAGAG R AACAGGAAGGCACTCAAGG	146
<i>S6K</i>	NM_001030721.1	F CAATTGCTCCCTACCTCA R AAGGAGGTTCCACCTTTCGT	176
<i>PKC</i>	NM_001012804.1	F GGCGGACAGGAAGAATACAGAGG R GAAGCTGTGTCAGGAATGGTGGTT	146
<i>avTOR</i>	XM_417614.6	F CACAACCACTGCTCGCCACAA R CCATAGGATCGCCACACGGATTAG	124
<i>β-actin</i>	NM_205518.1	F GAGAAATTGTGCGTGACATCA R CCTGAACCTCTCATTGCCA	152

Abbreviations: F, sense primers; R, antisense primers.

way ANOVA. Significant differences at the 0.05 level due to treatments were separated by Duncan's multiple range tests. Data were expressed as means \pm SD. Statistical significance was set at a level of $P < 0.05$.

RESULTS

Melatonin Enhances Melatonin Receptor Expression and Granulosa Cell Proliferation and Attenuates Granulosa Cell Apoptosis and Autophagy

Melatonin treatment significantly upregulated the mRNA and protein expression of MTR1 and MTR2 ($P < 0.01$) (Figures 2A and 2B). The mRNA levels of MTR1 and MTR2 in the 20- and 200- μ mol/L melatonin groups were significantly greater than those in the 2- μ mol/L melatonin and control groups ($P < 0.01$) and were significantly higher in the 2- μ mol/L melatonin group than in the control group ($P < 0.01$). Thus, the presence of melatonin increased the expression of melatonin receptors in the chicken granulosa cells. The mRNA results were confirmed by the MTR1 and MTR2 protein levels (Figures 2C–2E). The protein levels of MTR1 and MTR2 in the 20- and 200- μ mol/L melatonin groups were significantly higher than those in the 2- μ mol/L melatonin group and the control group ($P < 0.01$).

Cell proliferation and apoptosis after incubation with 2-, 20-, and 200- μ mol/L melatonin was determined. The

results showed that melatonin treatment increased cell proliferation at doses of 2 μ mol/L and 200 μ mol/L (Figure 3A). Flow cytometry was used to determine apoptosis (Figure 3E). The results showed that melatonin significantly increased the number of viable cells ($P < 0.05$) (Figure 3B) and reduced the number of apoptotic ($P < 0.05$) (Figure 3C) and necrotic cells ($P < 0.05$) (Figure 3D) compared with the control group. These results indicate that melatonin can inhibit the apoptosis of chicken granulosa cells in a concentration-dependent manner.

The mRNA and protein expression were analyzed by qRT-PCR and Western blot. The 20- and 200- μ mol/L melatonin treatments significantly increased the mRNA expression of cyclin D1 and decreased the mRNA expression of P21 compared to the control group ($P < 0.01$) (Figure 4A). Melatonin treatment significantly increased the mRNA expression of Bcl-2 ($P < 0.01$), and 20- or 200- μ mol/L melatonin significantly decreased the mRNA expression of caspase-3 ($P < 0.05$) (Figure 4B). The 20- and 200- μ mol/L melatonin treatments significantly decreased the mRNA expression of Beclin1 and LC3-II ($P < 0.01$) (Figure 4C). From the protein results, we found that the protein level of cyclin D1 was also elevated. The LC3I/LC3-II protein ratio declined with melatonin treatment, and these results were also confirmed by the protein levels of caspase-3 ($P < 0.01$) (Figures 4D–4G). Taken together, these results reveal that melatonin

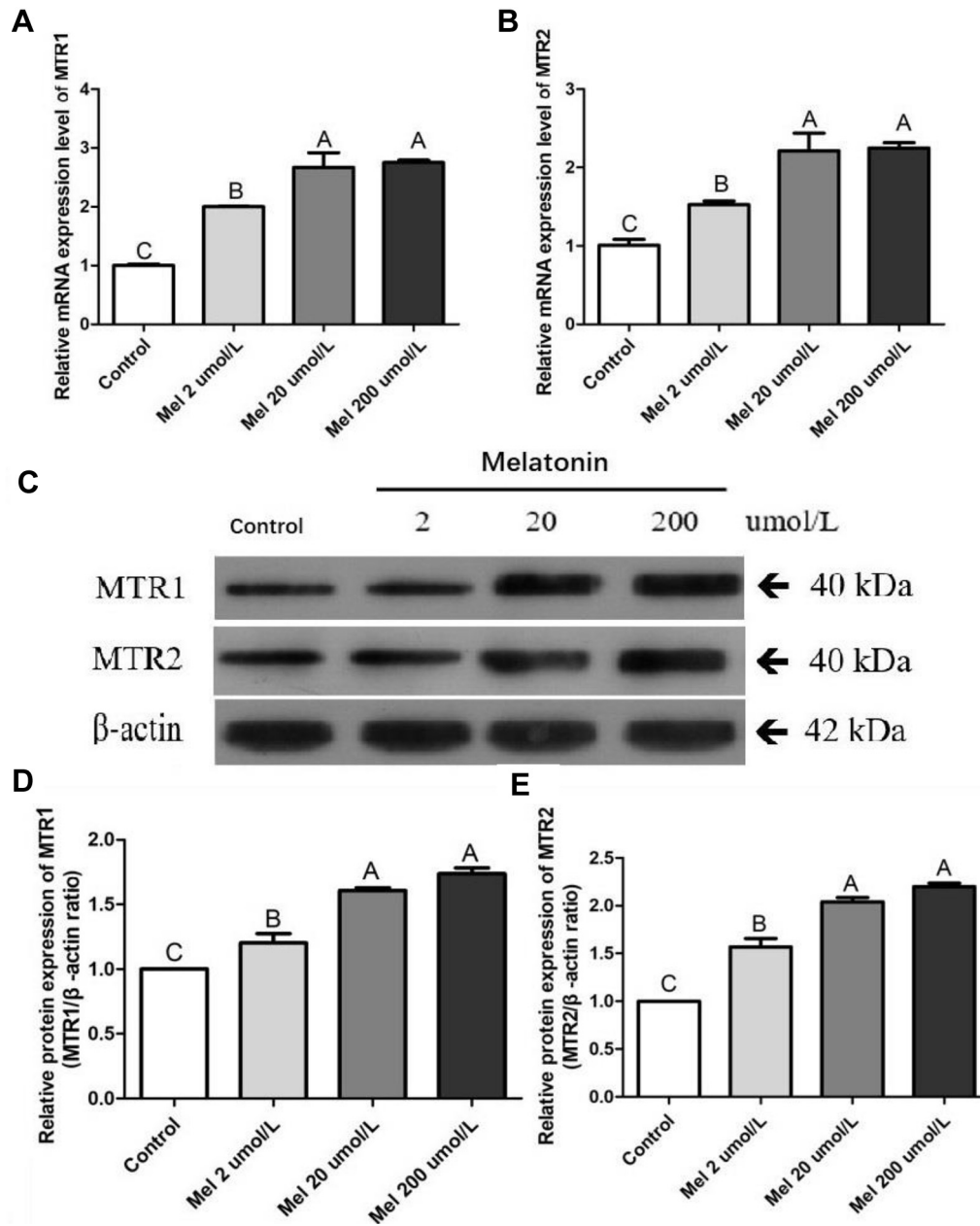


Figure 2. Effects of melatonin on MTR1 and MTR2 mRNA expression of chicken granulosa cells. Melatonin stimulated mRNA (A and B) of MTR1 and MTR2 in chicken granulosa cells. (C, D, and E) Melatonin stimulated protein expression of MTR1 and MTR2 in chicken granulosa cells. Bars (mean \pm SD) with different lowercase or uppercase letters are significantly different representing significant differences ($P < 0.05$).

could play a role in regulating the proliferation, apoptosis, and autophagy of chicken granulosa cells.

Melatonin Activates the mTOR Signaling Pathway

Melatonin treatment increased the mRNA expression of avTOR, PKC, 4E-BP1, and S6K, with the greatest effect seen at a concentration of 20 $\mu\text{mol/L}$ ($P < 0.01$) (Figure 5). The mRNA expression of avTOR in the 20- $\mu\text{mol/L}$ and 200- $\mu\text{mol/L}$ melatonin treatment groups showed a highly significant increase compared with the 2- $\mu\text{mol/L}$ melatonin and control groups ($P < 0.01$). PKC mRNA expression in the 20- $\mu\text{mol/L}$ melatonin treatment group was significantly increased compared

with the 2- $\mu\text{mol/L}$ and 200- $\mu\text{mol/L}$ melatonin treatment groups ($P < 0.05$) and highly significantly increased compared with the control group ($P < 0.01$). The mRNA expression of S6K and 4E-BP1 in the 20- $\mu\text{mol/L}$ and 200- $\mu\text{mol/L}$ melatonin treatment groups was significantly increased compared with that in the control group ($P < 0.01$). The expression of mTOR and 6SK and the phosphorylation of mTOR and S6K were also stimulated by melatonin (Figures 6A-6E). The protein expression of mTOR in the 200- $\mu\text{mol/L}$ melatonin treatment group was increased compared with that in the other groups ($P < 0.01$) (Figure 6B). The protein expression of p-mTOR and p-S6K in the melatonin treatment groups increased in a concentration-dependent manner compared with the

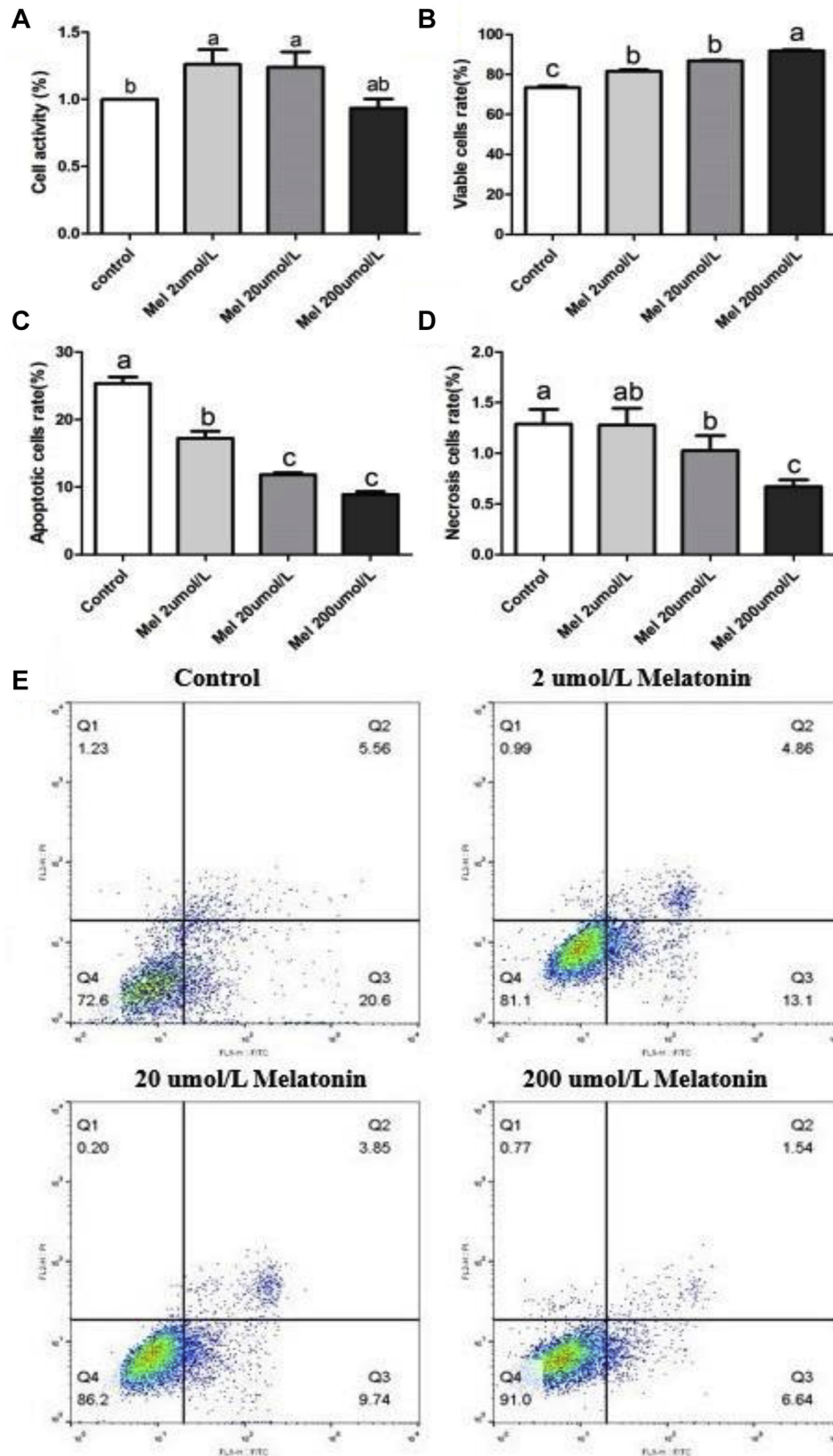


Figure 3. Effects of melatonin on proliferation and apoptosis of chicken granulosa cells. (A) Effects of melatonin on the chicken granulosa cell proliferation. In all panels, values represent the mean \pm SD based on 6 independent experiments. ($^{a-b}P < 0.05$ compared with the control group). (B, C, and D) Effects of melatonin on the chicken granulosa cell apoptosis rate. Flow cytometry images (E) and analysis of granulosa cell apoptosis at different melatonin concentrations. Annexin V-FITC/PI double staining analysis of apoptosis in chicken granulosa cells: Q1, necrotic cells; Q2, dead cells in the late stage of apoptosis; Q3, dead cells in the early stage of apoptosis; Q4, viable cells. In all panels, values represent the mean \pm SD based on 3 independent experiments. ($^{a-b}P < 0.05$ compared with the control group).

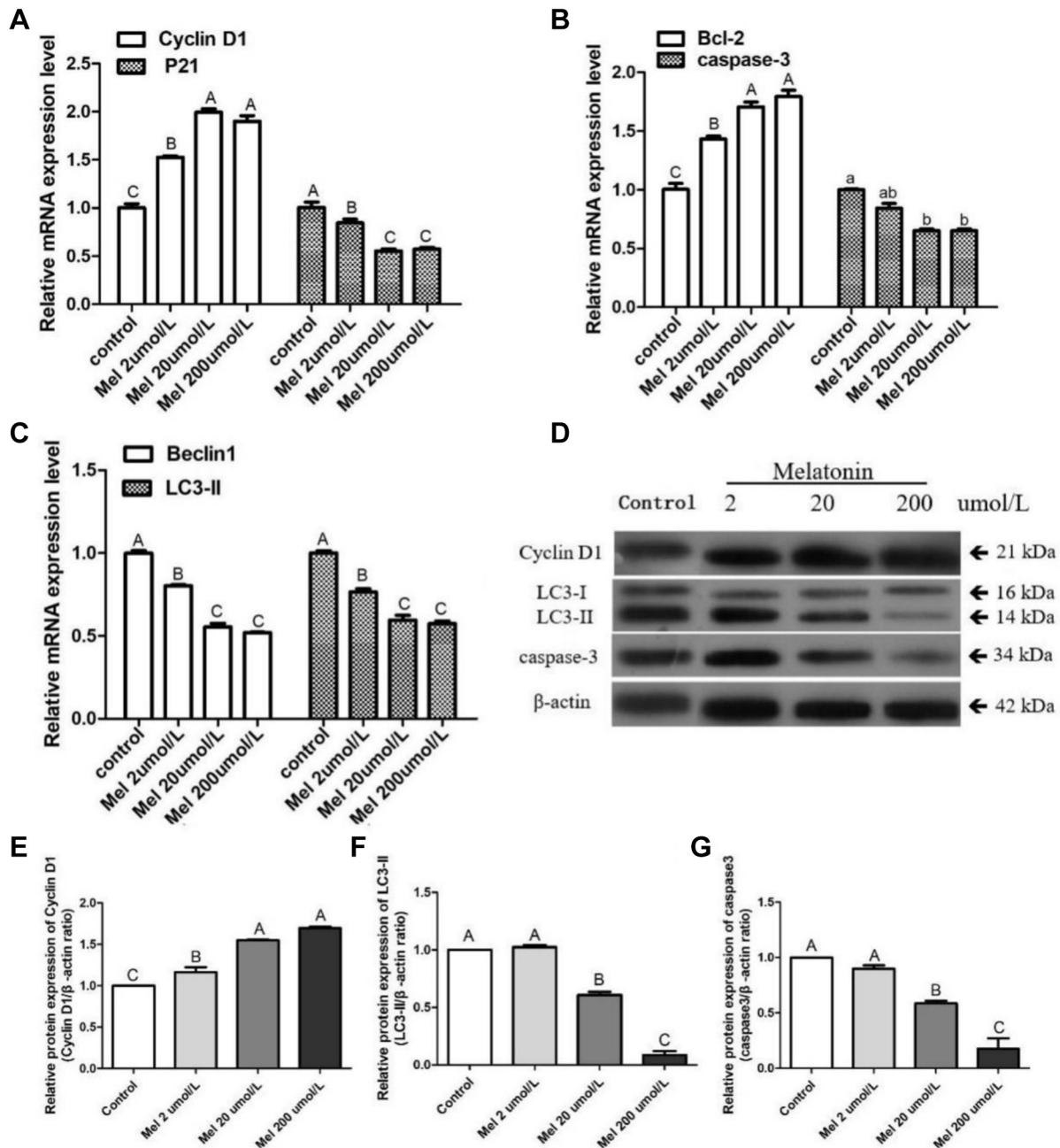


Figure 4. Effects of melatonin on mRNA and protein levels of cyclin D1 and p21, associated with cell proliferation, apoptosis, and autophagy genes mRNA and protein expression of chicken granulosa cells. (A) Effects of melatonin on the cyclin D1 and P21 mRNA expression, associated with cell proliferation. (B) Effects of melatonin on the Bcl-2 and caspase-3 mRNA expression, associated with cell apoptosis. (C) Effects of melatonin on the beclin1 and LC3-II mRNA expression, associated with cell autophagy. (D, E, F, and G) Effects of melatonin on the cyclin D1, LC3I/LC3-II, and caspase-3 protein expression. Changes in the cyclin D1, caspase-3, and LC3-I/LC3-II ratio can be seen in the Western blot. Bars (mean \pm SD) with different lowercase or uppercase letters are significantly different representing significant differences ($^{A-B}P < 0.01$).

control group ($P < 0.01$) (Figures 6C and 6E). These results reveal that the mTOR pathway was involved in the effect of melatonin on chicken granulosa cells.

The mTOR Pathway Is Involved in the Regulation of Melatonin-Mediated Proliferation, Apoptosis, and Autophagy of Chicken Granulosa Cells

MHY1485 promoted granulosa cell proliferation (Figure 7A), with the results showing an increased effect

of 10- μ mol/L MHY1485 on the cells compared with 1- μ mol/L MHY1485 and the control group ($P < 0.05$), while rapamycin significantly inhibited granulosa cell proliferation in the 100-nmol/L and 1,000-nmol/L groups compared with the control group ($P < 0.05$) (Figure 7B). Maximal effects were found at the concentrations of 10 μ mol/L of MHY1485 and 100 nmol/L rapamycin, so these concentrations were chosen for subsequent experiments. Granulosa cell proliferation and apoptosis were analyzed using the CCK-8 and TUNEL assays. In granulosa cells treated with either 20 μ mol/

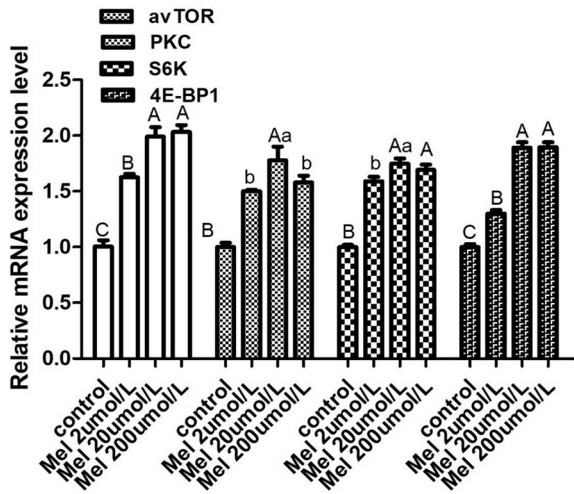


Figure 5. Effects of melatonin on the activation of mTOR signalling pathway in chicken granulosa cells. Expression of genes involved in the mTOR pathway. Bars (mean \pm SD) with different lowercase or uppercase letters are significantly different representing significant differences ($^{a-b}P < 0.05$, $^{A-B}P < 0.01$).

L melatonin or 20 $\mu\text{mol/L}$ melatonin + 10 $\mu\text{mol/L}$ MHY1485, proliferation was enhanced ($P < 0.05$) (Figure 7C). While rapamycin alone significantly inhibited cell proliferation ($P < 0.05$), in combination with 20 $\mu\text{mol/L}$ melatonin, it had no effect at the 100-nmol/L dose (Figure 7C). The TUNEL analysis revealed that 100 nmol/L rapamycin significantly enhanced both apoptosis and necrosis and inhibited the growth of viable cells ($P < 0.05$), but this effect was significantly attenuated by the presence of melatonin or the combination of 20 $\mu\text{mol/L}$ melatonin and 10 $\mu\text{mol/L}$ MHY1485 (Figures 7D–7G).

The mRNA levels and protein expression were determined by qRT-PCR and Western blot, respectively. We found that 20 $\mu\text{mol/L}$ melatonin together with 10 $\mu\text{mol/L}$ MHY1485 significantly upregulated the mRNA levels of cyclin D1 and Bcl-2 but decreased the mRNA expression of P21, caspase-3, Beclin1, and LC3-II compared to the control groups ($P < 0.05$). Rapamycin attenuated the mRNA levels of cyclin D1 and Bcl-2 and enhanced the mRNA levels of P21, caspase-3, Beclin1, and LC3-II, an effect that was reversed by the combination of 20 $\mu\text{mol/L}$ melatonin and 100 nmol/L rapamycin (Figures 8A–8C). Western blot analysis confirmed these results (Figures 8D–8G). The protein expression of LC3-II and caspase-3 in the 20- $\mu\text{mol/L}$ melatonin + 10 $\mu\text{mol/L}$ MHY1485 group was significantly decreased compared with that in the other group ($P < 0.01$), while the cyclin D1 level was increased compared with that in the other groups ($P < 0.01$). These results strongly confirm that melatonin exerts proliferative, antiapoptotic, and antiautophagic effects on chicken granulosa cells.

mTOR Pathway Involved in the Regulation of Melatonin-Mediated Genes Related to the mTOR Signaling Pathway of Chicken Granulosa Cells

Cells exposed to 20 $\mu\text{mol/L}$ melatonin and 10 $\mu\text{mol/L}$ MHY1485 showed increased mRNA expression of the avTOR pathway upstream genes PI3K, AKT1, and AKT2 and the downstream genes PKC, 4E-BP1, and S6K ($P < 0.05$) (Figure 9A). The maximum effect occurred with the combined treatment of 20 $\mu\text{mol/L}$ melatonin + 10 $\mu\text{mol/L}$ MHY1485. The combined treatment of 20 $\mu\text{mol/L}$ melatonin and 100 nmol/L rapamycin showed that melatonin prevented the rapamycin-mediated decrease in the mTOR pathway upstream genes PI3K, AKT1, and AKT2 and the downstream genes PKC, 4E-BP1, and S6K ($P < 0.05$) (Figure 9A). The protein levels of mTOR, p-mTOR, S6K, and p-S6K also confirmed the mRNA results (Figure 9B–9F). The protein expression of mTOR, p-mTOR, S6K, and p-S6K in 20 $\mu\text{mol/L}$ melatonin + 10 $\mu\text{mol/L}$ MHY1485 showed a highly significant upregulation and a highly significant downregulation in the 100-nmol/L rapamycin group ($P < 0.01$). These results strongly indicate that melatonin regulates granulosa cell proliferation, together with strong antiapoptosis and antiautophagy effects through activating the mTOR signaling pathway.

Effects of Activating and Blocking the mTOR Signaling Pathway on Melatonin Receptor Expression

The mRNA expression of MTR1 and MTR2 was significantly increased in the 20- $\mu\text{mol/L}$ melatonin group and the 10- $\mu\text{mol/L}$ MHY1485 group and also in the 20- $\mu\text{mol/L}$ melatonin + 10 $\mu\text{mol/L}$ MHY1485 group ($P < 0.01$) (Figures 10A and 10B). Pharmacological blockage of the mTOR pathway (by 100 nmol/L rapamycin) significantly decreased MTR1 and MTR2 gene expression ($P < 0.05$). The same result was also detected in the Western blot (Figures 10C–10E). The protein expression of MTR1 and MTR2 in the 20- $\mu\text{mol/L}$ melatonin + 10 $\mu\text{mol/L}$ MHY1485 group was significantly increased compared with that in the other groups ($P < 0.01$). The protein expression of MTR1 and MTR2 in the 20- $\mu\text{mol/L}$ melatonin group, 10- $\mu\text{mol/L}$ MHY1485 group, and the 20- $\mu\text{mol/L}$ melatonin + 100-nmol/L rapamycin group were significantly increased compared with the control group and the 100-nmol/L rapamycin group ($P < 0.01$).

DISCUSSION

The ovaries of birds contain different sizes of follicles. Research has found that the growth of follicles involves an increase in the shape and number of granulosa cells

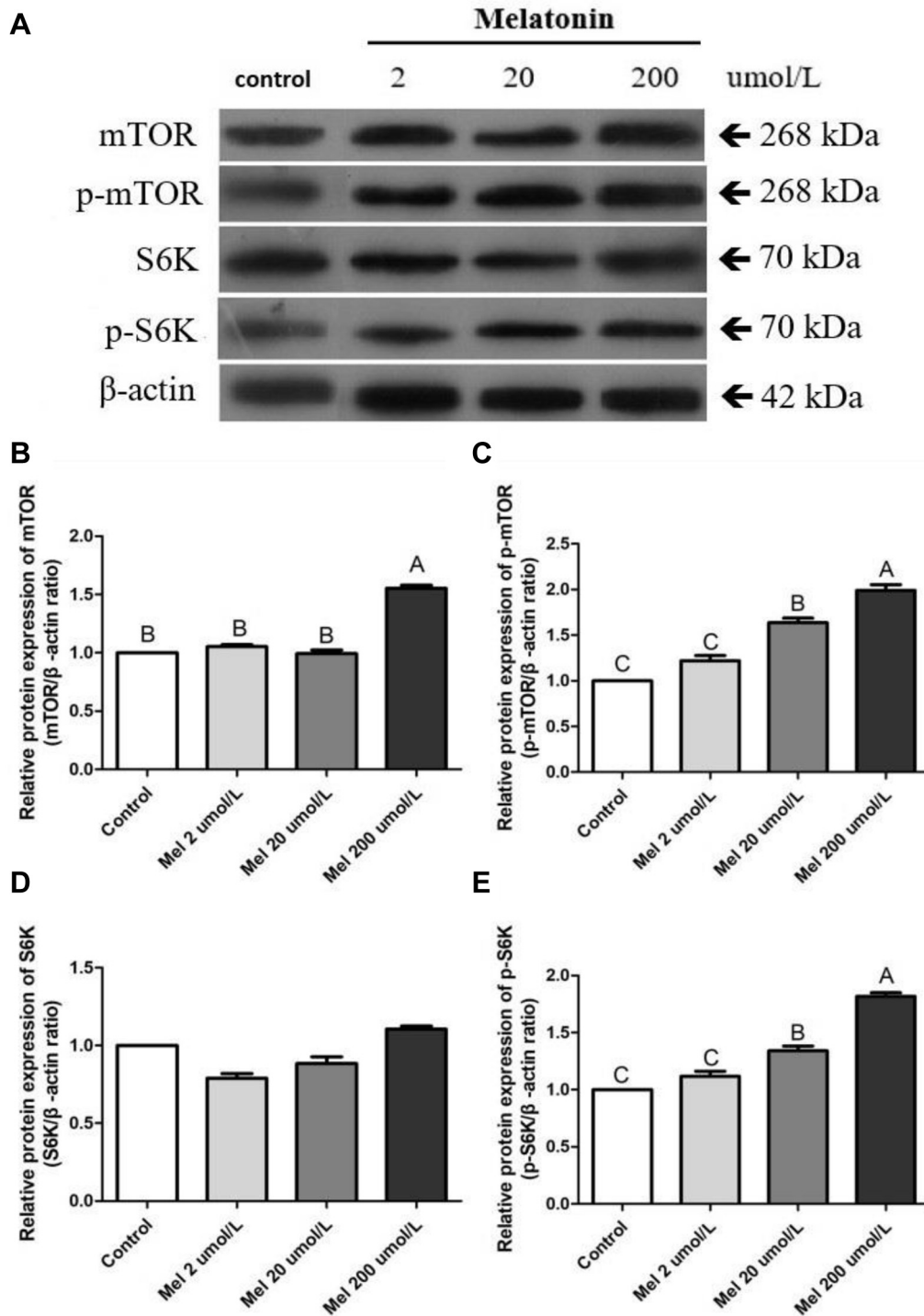


Figure 6. Effects of melatonin on the activation of mTOR signalling pathway in chicken granulosa cells. (A-E) Influence of melatonin on the protein expression of mTOR, phosphorylation of mTOR, S6K, and phosphorylation of S6K. Bars (mean \pm SD) within each grouping with different lowercase or uppercase letters are significantly different from each other ($A-B P < 0.01$).

(Braw-Tal, 2002). As granulosa cell proliferation represents follicular development, we hypothesized that melatonin may regulate follicular development by enhancing the proliferation of these granulosa cells. In the present study, we found that melatonin treatment significantly enhanced the proliferation of chicken granulosa cells. Treatment with 20 $\mu\text{mol/L}$ melatonin and 200 $\mu\text{mol/L}$ melatonin increased the mRNA expression of cyclin D1 and decreased the expression of P21. The cell cycle

gene cyclin D1 plays a key role in the cell cycle process from G1 (pre-DNA synthesis) to S (DNA synthesis period) (Sherr, 1995). P21 is an inhibitor of cyclin-dependent kinases and inhibits the cell cycle from G1 (pre-DNA synthesis) to S (DNA synthesis period) and G2 (late DNA synthesis) to M (cell division) (Harada, 2000). Melatonin at 10^{-9} mol is known to significantly increase the proliferation of T-lymphocytes in rats (Chen, 2016). Our results are also consistent with other

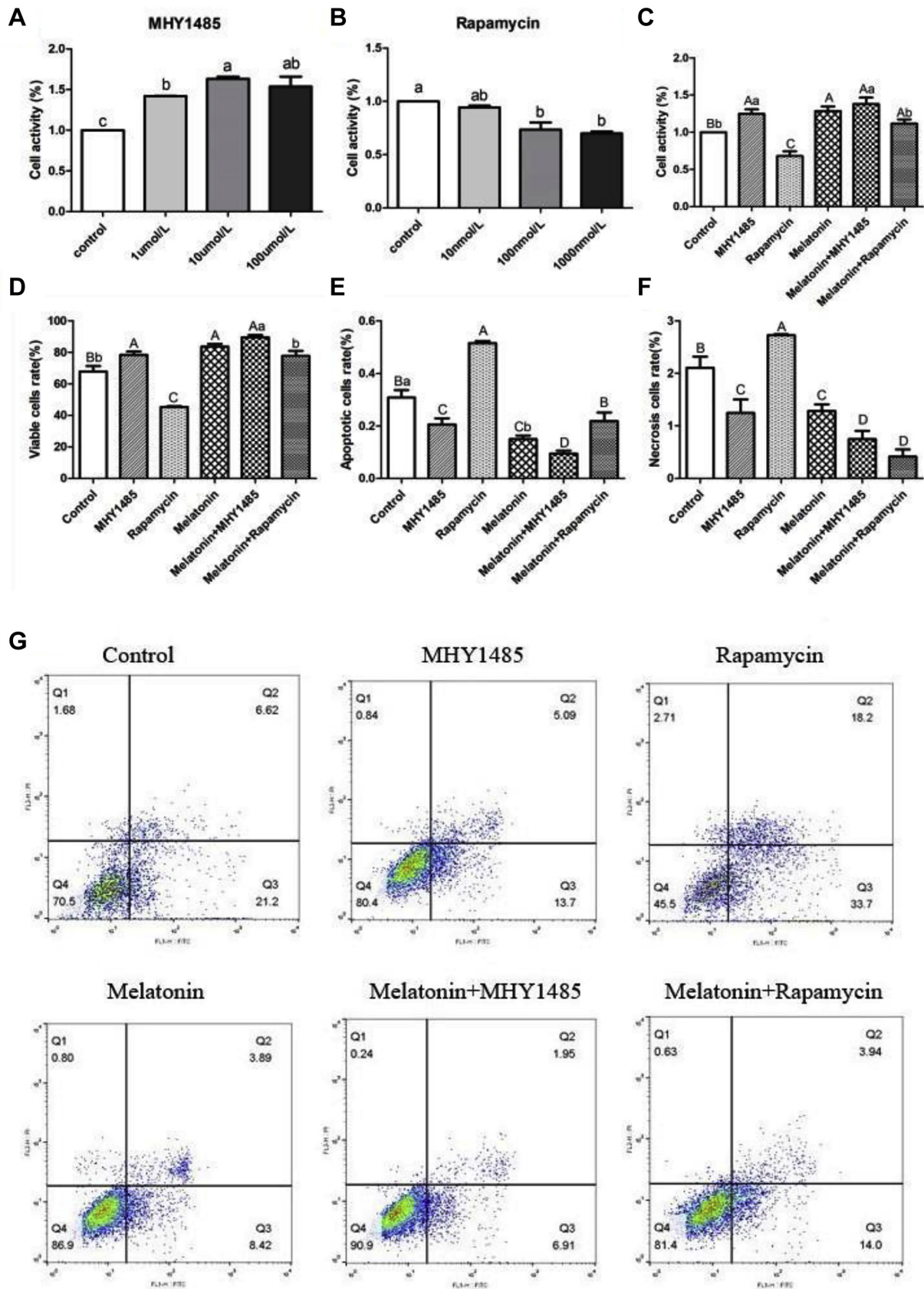


Figure 7. Activation and blockade of the mTOR pathway regulates melatonin-induced effects on proliferation and apoptosis of chicken granulosa cells. (A) Effect of the mTOR activator (MHY1485) on the proliferation of granulosa cells. (B) Effect of an mTOR inhibitor (rapamycin) on the proliferation of granulosa cells. (C) Effects of melatonin in combination with either MHY1485 (10 μ mol/L) or rapamycin (100 nmol/L) on the proliferation of granulosa cells. (D, E, and F) Effects of melatonin in combination with either MHY1485 (10 μ mol/L) or rapamycin (100 nmol/L) on cell apoptosis analyzed by the TUNEL method. Flow cytometry images (G) of the chicken granulosa cells apoptosis at different concentration melatonin. Annexin V-FITC/PI double staining analysis of apoptosis in chicken granulosa cells. Q1: necrotic cells; Q2: dead cells in the late stage of apoptosis; Q3: dead cells in the early stage of apoptosis; Q4: viable cells. (^{a-b} $P < 0.05$, ^{A-B} $P < 0.01$).

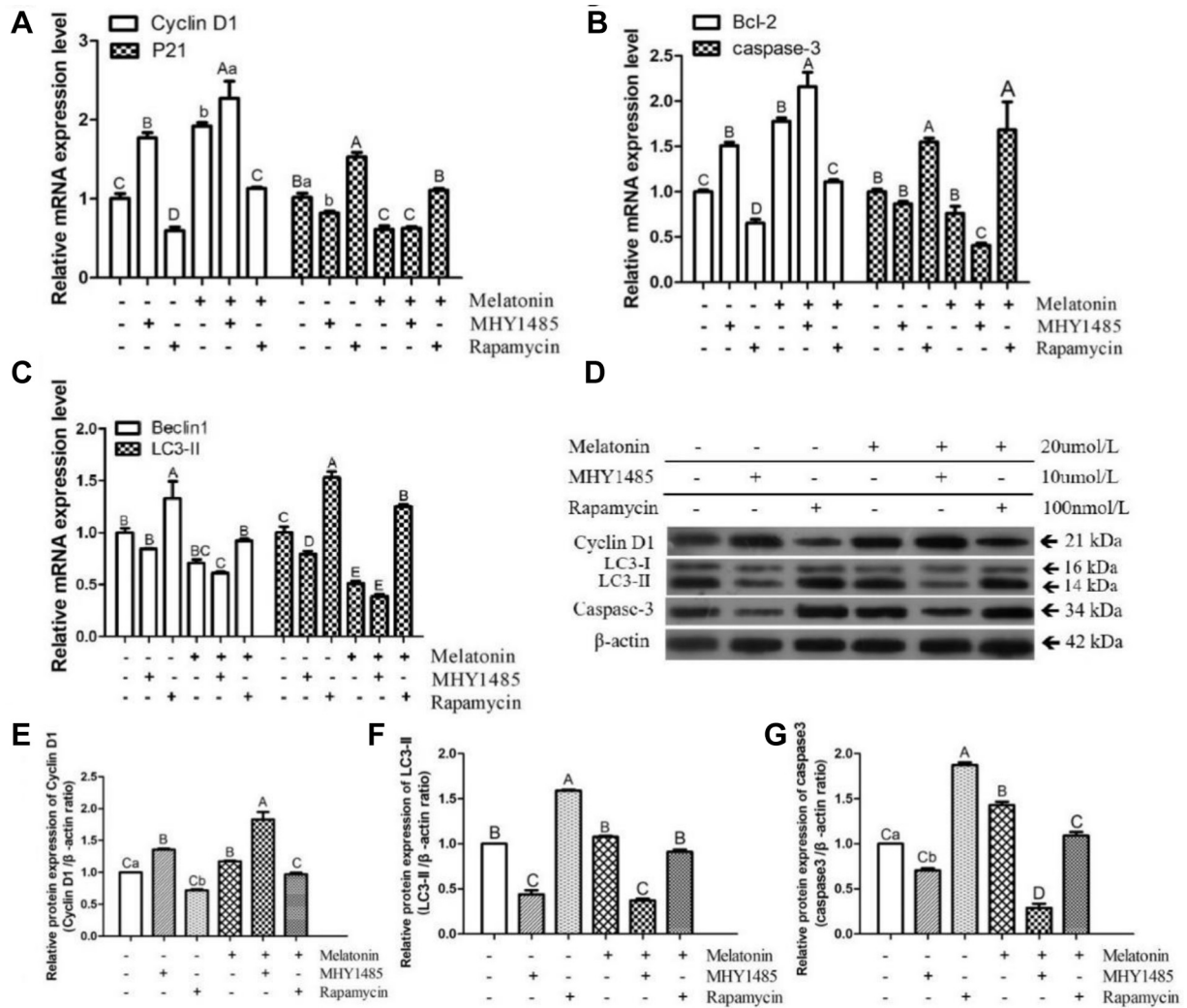


Figure 8. Activation and blockade of the mTOR pathway regulates melatonin-induced effects on proliferation and apoptosis of chicken granulosa cells. Effects of melatonin in combination with either MHY1485 (10 μmol/L) or rapamycin (100 nmol/L) on the mRNA levels (A-C) and protein expression (D-G) of selected genes associated with cell proliferation, apoptosis, and autophagy. Differences in band densities of cyclin D1, LC3I/LC3-II, and caspase-3 were determined by densitometry and plotted. Bars (mean ± SD) within each grouping with different lowercase and uppercase letters are significantly different from each other (^{a-b}*P* < 0.05, ^{A-B}*P* < 0.01).

research in sheep granulosa cells (Fu, 2014) and human bone cells (Nakade, 2010). Therefore, we propose that melatonin could enhance cell proliferation by activating cyclin D1 and P21.

We used TUNEL analysis to detect the effects of melatonin treatment on apoptosis in chicken granulosa cells and found that melatonin decreased the granulosa cell apoptosis rate in a concentration-dependent manner. Moreover, our results found that melatonin treatment significantly increased the mRNA expression of the anti-apoptotic marker Bcl-2 (Kroemer, 1997) and decreased that of the proapoptotic marker caspase-3 (Porter, 1999). Follicular atresia is an apoptotic process that is regulated by proapoptotic factors and antiapoptotic factors. The internal and external signals of follicles determine whether follicles continue to develop or atrophy. The Bcl2 family is important in regulating follicular atresia and apoptosis. The number of atretic follicles in wild-type rats has been found to be significantly lower

than that in Bcl2 knockdown rats, meaning that overexpression of granulocyte Bcl2 can significantly reduce the occurrence of apoptosis (Jones, 2014). A previous study showed that in rats with knockout caspase3 gene, apoptosis was significantly inhibited and follicular atresia was also reduced. Caspase-3 plays a key role in cell apoptosis, and its activation can cause irreversible apoptosis (Earnshaw, 1999; Porter, 1999). Another study showed that caspase-3 knockout causes cells to undergo irreversible apoptosis (Matikainen, 2001). Melatonin can inhibit mitochondrial pathway-mediated apoptosis by inducing Bcl2 expression and decreasing caspase-3 activity (Matikainen, 2001). Melatonin also suppressed caspase-dependent apoptosis by increasing expression of Bcl-2 and Bax, thus reducing oxidative stress (Wang and Zeng, 2018). After treatment with melatonin, the protein expression of caspase-3 is also significantly lowered, while the expression of Bcl-2 is raised in cells from rats with diabetic retinopathy (Ma

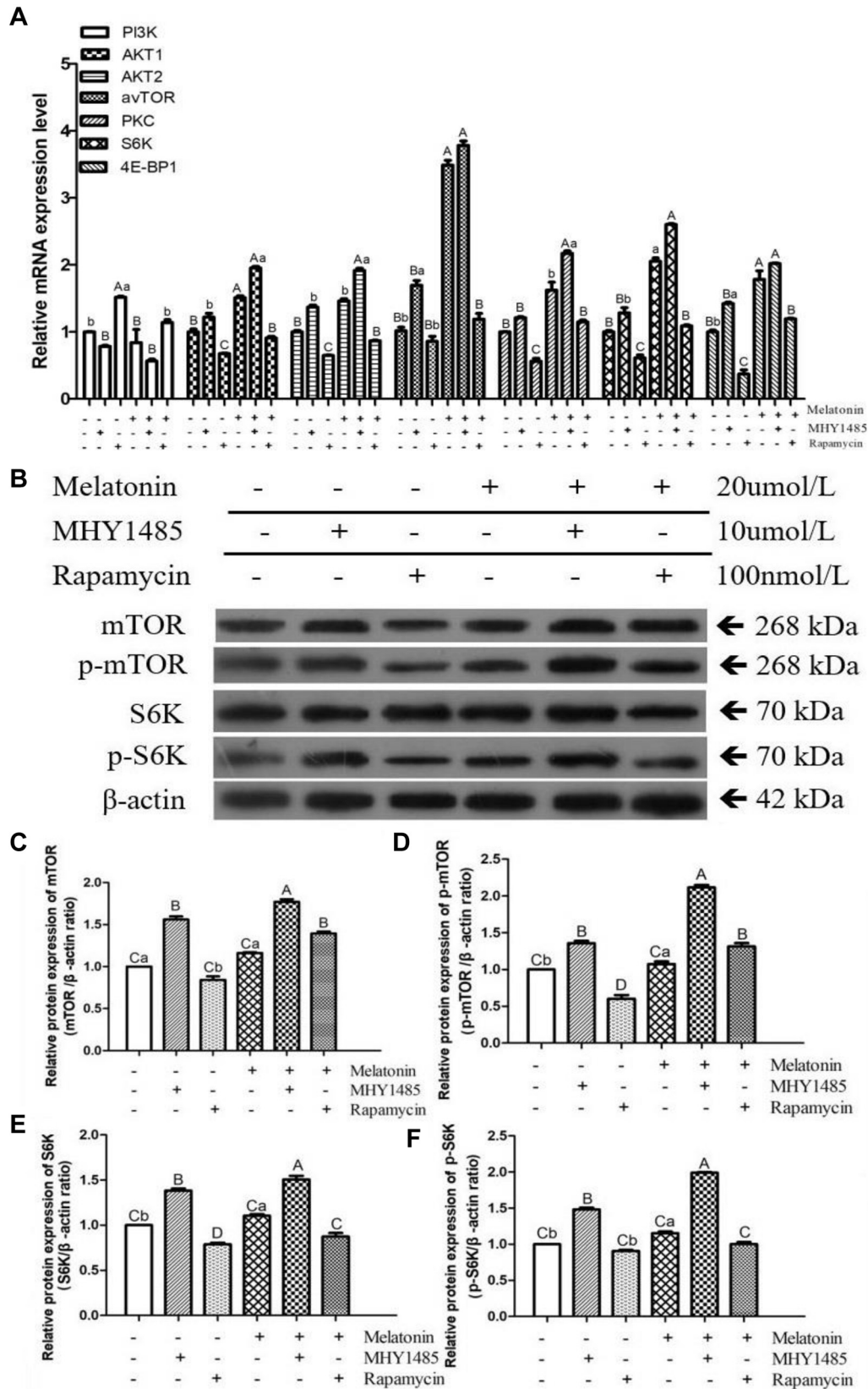


Figure 9. Activation and blockade of the mTOR pathway regulates melatonin-induced effects on proliferation and apoptosis of chicken granulosa cells. (A) Effects of melatonin in combination with either MHY1485 (10 $\mu\text{mol/L}$) or rapamycin (100 nmol/L) on the mRNA levels of selected genes associated with regulation of the PI3K/Akt/mTOR pathway. (B-F) Effects of melatonin in combination with either MHY1485 (10 $\mu\text{mol/L}$) or rapamycin (100 nmol/L) on the protein expression of selected genes associated with regulation of the PI3K/Akt/mTOR pathway. Bars (mean \pm SD) with different lowercase and uppercase letters are significantly different ($^{a-b}P < 0.05$, $^{A-B}P < 0.01$).

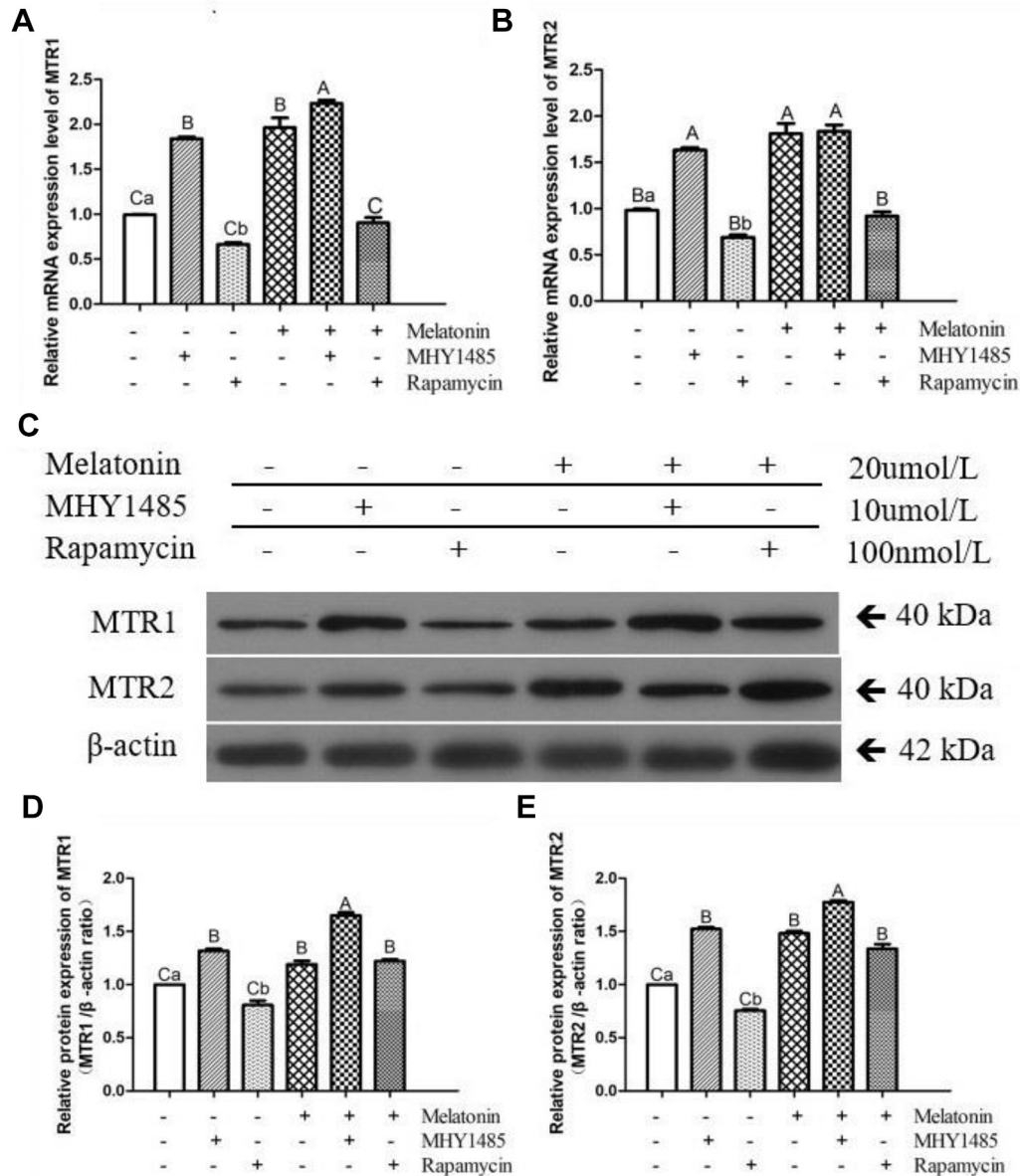


Figure 10. Effects of melatonin on expression of MTR1 and MTR2 within chicken granulosa cells in the presence of either MHY1485 or rapamycin. (A and B) MTR1 and MTR2 mRNA levels were determined by qRT-PCR. (C, D, and E) MTR1 and MTR2 protein expression were determined by Western Blot. Bars (mean \pm SD) with different lowercase and uppercase letters are significantly different ($^{a-b}P < 0.05$, $^{A-B}P < 0.01$).

et al., 2019). The results of both these studies are consistent with our findings. Thus, exogenous melatonin can reduce the apoptosis rate of chicken granulosa cells.

In the present study, we found that 20- and 200- μ mol/L melatonin significantly downregulated the mRNA levels of Beclin1 and LC3-II. Autophagy is important for cell survival, differentiation, and homeostasis. Beclin-1 is a specific marker of autophagic cell death (Yasuko, 2005). Kabeya et al. (Kabeya, 2000) reported that LC3-II is a marker of the autophagic process. The level of LC3-I/LC3-II is an indicator of autophagic activity. Kim found that melatonin (100 μ mol/L) inhibited apoptotic and autophagic cell death by increasing the expression of Bcl-2 and reducing LC3-II expression in C2C12 murine myoblast cells (Kim et al., 2011). Research has also found that melatonin (100 and 200 nmol/L) significantly attenuated LC3 II protein

expression in clinostat-treated cells (Bennukul, 2014). These findings are consistent with our Western blot results. The protein expression of cyclin D1 was increased, and the caspase-3 and LC3I/LC3-II ratio was downregulated by 20- μ mol/L melatonin treatment in chicken granulosa cells. Taken together, it is clear that melatonin can regulate proliferation and has antiapoptosis and anti-autophagy effects in chicken ovarian granulosa cells.

The mTOR signaling pathway is important in the regulation of cellular growth, such as proliferation, differentiation, survival, and metabolism (Schmelzle, 2000). This present study explored a connection between melatonin and the mTOR signaling pathway. The results showed that melatonin activated mTOR signaling in chicken granulosa cells. The mTOR pathway downstream expression of mTOR and the PKC, 4E-BP1, and S6K genes was upregulated. Protein expression

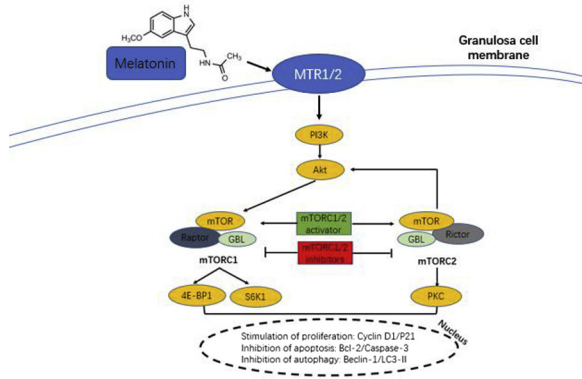


Figure 11. Proposed mechanism by which melatonin exerts its proliferative and antiapoptotic effects on chicken granulosa cells. Briefly, interaction between melatonin and its receptor promotes PI3K to generate inositol phospholipids, as the second messengers activate the phosphorylation of Akt. Akt subsequently activates the mTOR-raptor complex, thereby resulting in the phosphorylation of many target proteins and finally regulating proliferation and apoptosis of chicken granulosa cells. MTR1/2 represents melatonin receptors.

results of mTOR, phosphorylation of mTOR and S6K, and phosphorylation of S6K also confirmed the activation of the mTOR signaling pathway. Previous research found that melatonin (0.5, 1, 5, and 10 $\mu\text{mol/L}$) significantly increased the mTOR and p-mTOR protein expression by activation of the mTOR pathway in high-glucose-treated Schwann cells (Tiong, 2019). Koh found that melatonin inhibited ischemic brain injury through activation of the mTOR signaling pathway (Koh, 2008). These studies are all in agreement with our study. The mTOR signaling pathway is regulated by the upstream PI3K-Akt signaling pathway (Ma and Blenis., 2009; Ben, 2010). Melatonin can activate the PI3K/Akt pathway, and the activated Akt stimulates mTOR phosphorylation (Kim et al., 2014; Kilic, 2017). In addition, phosphorylation of mTOR activates the downstream target proteins 4E-BP1 and p70S6K to promote cell growth and protein synthesis (Burgos, 2010). Furthermore, in the present study, the mTOR inhibitor (rapamycin) and mTOR agonist (MHY1485) were used to determine whether the mTOR signaling pathway is involved in melatonin-mediated cell growth in chicken granulosa cells. It was found that cells exposed to 20 $\mu\text{mol/L}$ melatonin and 10 $\mu\text{mol/L}$ MHY1485 showed proproliferation and antiapoptosis effects and significantly increased the expression of the mTOR pathway upstream genes PI3K, AKT1, and AKT2 and downstream genes PKC, 4E-BP1, and S6K and protein expression of p-mTOR and p-S6K. The 100-nmol/L rapamycin significantly attenuated the effects of melatonin on granulosa cells and apoptosis as well as the expression of genes associated with cell proliferation, apoptosis, and mTOR signaling. Combined treatment (20 $\mu\text{mol/L}$ melatonin and 100 nmol/L rapamycin) showed that melatonin prevented rapamycin from decreasing the mTOR pathway upstream genes PI3K, AKT1, and AKT2 and the downstream genes of PKC, 4E-BP1, and S6K and protein expression of p-mTOR

and p-S6K. Taken together, these results reveal that the mTOR signaling pathway is involved in melatonin-mediated effects on chicken granulosa cell function.

Melatonin exerts its biological actions through both specific transmembrane G-protein-coupled receptor-mediated and independent-receptor mechanisms (Pandi-Perumal, 2008). Melatonin receptors have been found in the brain (Rivkees, 1989), ovary, lungs, spleen, and kidneys of the chicken (Pang, 1995; Sundaresan, 2009). Moreover, the presence of melatonin receptors in chicken granulosa cells has been confirmed (Murayama, 1997).

In the present study, we further demonstrated that melatonin has a stimulatory effect on the mRNA expression of MTR1 and MTR2. In addition, the mRNA expression levels of MTR1 and MTR2 were significantly enhanced by 20- $\mu\text{mol/L}$ melatonin and 10- $\mu\text{mol/L}$ MHY1485 and also in the 20- $\mu\text{mol/L}$ melatonin + 10 $\mu\text{mol/L}$ MHY1485 group. In addition, pharmacological block of the mTOR pathway significantly decreased MTR1 and MTR2 gene expression. However, the inhibitory effects of rapamycin on melatonin receptor MTR1 and MTR2 expression were significantly improved by the combination of 20 $\mu\text{mol/L}$ melatonin + 100 nmol/L rapamycin. Melatonin can activate different second messenger cascades by interacting with different receptor subtypes organized in different ways (Tamura et al., 2009). Research has reported that melatonin attenuated milk fat synthesis by inhibiting the mTOR signaling pathway via the MT1 receptor in brain microvascular endothelial cells (Wang et al., 2012). Melatonin downregulates autophagy and exerts protective effects via activation of mTOR signaling in liver ischemia-reperfusion injuries (Kang et al., 2014). Taken together, it is likely that melatonin regulates the mTOR signaling pathway through the melatonin receptor in chicken granulosa cells.

CONCLUSION

In conclusion, our results revealed that melatonin regulates chicken granulosa cells' proliferative and antiapoptotic actions by activating the mTOR signaling pathway via its receptor (summarized in Figure 11). The results of this study will help us better understand the mechanism by which melatonin regulates follicles. Further studies should determine whether it is possible to increase the number of follicles by adding melatonin to regulate granulosa cell proliferation and inhibit apoptosis, thereby sustaining the egg production rate in older hens.

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