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# Effect of melatonin on *ATG2B*-mediated autophagy regulation in sheep granulosa cells with different $Fec^B$ genotypes

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## Abstract

Melatonin (MLT) protects cells by reducing reactive oxygen species (ROS) levels, which are key for inducing cellular autophagy. The aim of this study was to investigate the molecular mechanisms underlying MLT regulation of autophagy in granulosa cells (GCs) with *BMPR-1B* homozygous ($Fec^B$ BB) and wild type ($Fec^B$ ++) mutations. GCs collected from small-tailed Han sheep with different $Fec^B$ genotypes were typed using a TaqMan probe assay, and autophagy levels were found to be significantly higher in GCs with $Fec^B$ BB than the levels in those with $Fec^B$ ++. Autophagy-related 2 homolog B (*ATG2B*) was associated with cell autophagy and was highly expressed in GCs with the $Fec^B$ BB genotype in small-tailed Han sheep. Overexpression of *ATG2B* in the GCs of sheep with both $Fec^B$ genotypes promoted GC autophagy, and the contrary was observed after the inhibition of *ATG2B* expression. Subsequently, treatment of GCs with different genotypes of $Fec^B$ and MLT revealed a significant decrease in cellular autophagy and an increase in *ATG2B* expression. Addition of MLT to GCs with inhibited *ATG2B* expression revealed that MLT could protect GCs by decreasing ROS levels, especially in GCs with $Fec^B$ ++ genotype. In conclusion, this study determined that autophagy levels were significantly higher in sheep GCs with $Fec^B$ BB genotype than the levels in those with $Fec^B$ ++ genotype, which may have contributed to the difference in lambing numbers between the two $Fec^B$ genotypes. Autophagy was regulated by *ATG2B* and was able to protect GCs by reducing the high levels of ROS produced following inhibition of *ATG2B* through the addition of MLT in vitro.

**KEYWORDS**
*ATG2B*, autophagy, $Fec^B$ gene, granulosa cells, melatonin, sheep

Yu-Fang Liu and Zi-Yi Liu are equally contributed to this study.

## 1 | INTRODUCTION

Sheep are a crucial livestock species that provide meat, milk, fur, and wool to humans worldwide. Increasing the number of lambs produced by sheep is fundamental to the availability of sheep products. *Fec*$^B$ is a major effect gene associated with the lambing number trait in sheep, which has been used as a crucial molecular marker for selecting high-fertility sheep.[1–6] Studies have shown that *Fec*$^B$ has an additive effect on the ovulation rate, with effects on litter size ranging from additive to dominant depending on the genotype.[7] However, the molecular mechanism of *Fec*$^B$ mutation in the regulation of lambing number traits in sheep remains unclear.

The ovary is an important organ of the female reproductive system that plays a vital role in the growth and development of follicles.[8] Ovarian granulosa cells (GCs) wrap around the surface of oocytes, participate in follicular growth and development, and maintain ovarian function by secreting steroid hormones.[9] GCs proliferate and gradually differentiate during follicular development, supporting oocyte maturation and ovulation.[10] Few studies have shown that follicular atresia is caused by a decrease in follicle number due to apoptosis of GCs.[11,12] Cells maintain cellular energy homeostasis and function through autophagy and control the fate of cells through various signaling pathways. Meanwhile, it is also considered as a programmed cell death, namely type II programmed cell death.[13] Autophagy has a cytoprotective effect under certain stress conditions and can reduce cell death by inhibiting apoptosis.[14] In our previous single-cell sequencing study, we found that autophagy-related 2 homolog B (*ATG2B)* was significantly more highly expressed in the *Fec*$^B$ BB genotype than that in the *Fec*$^B$ ++ genotype of small-tailed cold sheep GCs. *ATG2B* is a member of the autophagy-related gene (ATG) family, which is usually upregulated in the presence of enhanced autophagic activity and has a function similar to that of the ATG family.[15] However, the mechanism through which *ATG2B* affects cellular autophagy requires further investigation.

Melatonin (MLT) is a well-known potent free radical scavenger and broad-spectrum antioxidant that protects normal cells.[16] During follicular development, increased levels of reactive oxygen species (ROS) are associated with an accelerated metabolic rate of rapid GC proliferation.[17] The accumulation of ROS induces oxidative damage to ovarian GCs, thereby initiating an atretic program that leads to the development of anovulatory disorders, such as polycystic ovary syndrome and premature ovarian failure.[18] Normal production of ROS in mammalian cells usually exhibits basic autophagic activity, whereas excessive autophagy induces self-destruction of cells suffering from oxidative damage.[19] Recent evidence suggests that MLT inhibits cellular autophagy through its antioxidant properties.[20,21] Further studies have revealed that the MLT-mediated inhibition of autophagic cell death increases cellular resistance to harmful stimuli.[22,23] In addition, one study found that the inhibition of FOXO1 by the MLT-phosphatidylinositol 3-kinase-AKT axis under oxidative stress conditions not only increases the resistance of GCs to oxidative stress but also eliminates the autophagic response.[24] Thus, whether MLT can affect GC autophagy by regulating ATG2B and the elucidation of this mechanism are crucial for ovulation in *Fec*$^B$ BB and ++ genotypes of small-tailed Han sheep.

In this study, we present a new pathway to reveal the molecular mechanisms underlying GC formation in different *Fec*$^B$ genotypes. This study provides a novel perspective for resolving the mechanisms that cause differences in ovulation numbers among individuals with different *Fec*$^B$ genotypes in sheep.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethical statement

All animal experiments were authorized by the Department of Scientific Research (responsible for animal welfare), Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS; No. IAS2020-82).

### 2.2 | Cell collection and culture in vitro

A total of 200 small-tailed Han sheep with lambing records of more than two litters were selected for *FecB* genotyping using the TaqMan probe method. All experiments involved sheep that were consistent in terms of weight, age and feeding environment. Sheep were treated with a controlled internal drug-releasing plug (CIDR, progesterone 300 mg, Inter Ag Co., Ltd.) for estrus synchronization for 12 d. Sheep were euthanized within 45–48 h of CIDR removal (follicular phase, FP). Immediately after euthanasia, ovarian tissues were collected and brought back to the laboratory for the collection of GCs.

Collected healthy sheep ovaries were stored in phosphate-buffered saline (PBS) containing 1% penicillin–streptomycin solution (100 IU/mL penicillin and 50 mg/mL streptomycin) and transported to the laboratory at 4°C. Primary ovarian GCs were isolated from sheep ovaries, according to a previously described method.[25] Sheep ovarian GCs and HEK293T cell line (Human Embryonic Kidney 293 Cells, which is a very

commonly used cell line for expression analysis of exogenous genes and purchased from COBIOER) were cultured at 37°C, 5% $CO_2$, and 95% humidity. Ovarian GCs were cultured in DMEM/F-12 medium (GIBCO, Thermo Fisher, Scientific) supplemented with 10% fetal bovine serum (GIBCO) and 100 U/mL penicillin/streptomycin (GIBCO).

## 2.3 | TaqMan probe analysis

TaqMan real-time polymerase chain reaction (PCR) was performed using primers and probes (Table 1) designed to identify single nucleotide polymorphisms at the $Fec^B$ locus. The primers and probes were synthesized by RuiBiotech. TaqMan real-time PCR (RT-PCR) reaction mixtures (20 μL) contained 10 μL Probe qPCR SuperMix (Tansgen), 1 μL forward primer (10 μmol/L), 1 μL reversed primer (10 μmol/L), 1 μL forward probe (10 μmol/L), 1 μL reversed probe (10 μmol/L), 1 μL of template (100 ng/μL), and 5 μL of $ddH_2O$. The RT-PCR reactions were performed under the following procedures: 30 s at 94°C, 40 cycles of 5 s at 94°C, and 30 s at 60°C (Roche LightCycler® 480 II System; Roche Applied Science).

## 2.4 | Immunofluorescence analysis

Isolated cells were identified as GCs using an immunofluorescence assay with FSHR (1:500; Bioss). Experiments were performed according to the manufacturer's instructions.

## 2.5 | Detection of ROS levels

ROS levels were determined using a ROS Assay Kit (Beyotime Institute of Biotechnology, S0033) according to the manufacturer's instructions. The fluorescence intensity of the GCs was calculated using ImageJ 1.42q software (National Institutes of Health).

## 2.6 | Enzyme-linked immunosorbent assay (ELISA)

The levels of estrogen ($E_2$), progesterone (PROG) and MLT were measured in GCs after different treatments according to the protocols of the $E_2$, PROG and MLT ELISA kits (Enzyme-linked Biotechnology Co., Ltd.), respectively. The absorbance was measured at 450 nm using a Fluorescence/Multi-Detection Microplate Reader (Bio-Rad).

## 2.7 | Real-time PCR (RT-qPCR)

RT-qPCR was used to verify the expression levels of *ATG2B* as well as markers of apoptosis, proliferation, and autophagy. *GAPDH* was used as an endogenous control to normalize gene expression. Primers were designed using Primer 5 (Supporting Information: Table S1). RT-qPCR was performed on a LightCycler 480 II (Roche) using SYBR Premix Ex Taq II. The cycling conditions were predenaturation at 95°C for 5 s, 40 cycles of 95°C for 5 s, and 60°C for 30 s. Melting curve analysis was performed, and the relative expression levels were determined using the $2^{-\Delta\Delta C_t}$ method.[26] All experiments were performed with three biological replicates, and each sample was tested in triplicate.

## 2.8 | Western blot analysis assay

Proteins were extracted from sheep ovarian tissues and GCs using a Total Protein Extraction Kit (Beyotime Biotech Co., Ltd.), and protein concentrations were determined using a BCA protein assay kit (Beyotime). The primary antibodies used were anti-ATG2B (1:1000) (Santa Cruz Biotechnology), anti-P62 (1:5000), anti-LC3 (1:500), anti-ATG7 (1:1000), and anti-GAPDH (1:1000) (all from Cell Signaling Technology). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L) (Proteintech), which was used at the dilution recommended by Beyotime.

**TABLE 1** Primer information for TaqMan assay.

| Primer name | Sequence (5′−3′) | Tm (°C) |
|---|---|---|
| $Fec^B$-primer | F: 5′-CCAGCTGGTTCCGAGAGACA-3′ | 60 |
| | R: 5′-CTTATACTCACCCAAGATGTTTTCATG-3′ | |
| $Fec^B$-probe | F: 5′-FAM-AAATATATCGGACGGTGTT-MGB-3′ | 60 |
| | R: 5′-HEX-AAATATATCAGACGGTGTTG-MGB-3′ | |

## 2.9 | Plasmid construction and cell transfection

The coding region of *ATG2B* was cloned into pcDNA3.1 expression vector, and the recombinant vectors were named pcDNA3.1-ATG2B and pcDNA3.1-NC. The siRNA- ATG2B and siRNA-NC were synthesized by Genewiz (Suzhou, China).

Sheep primary GCs were seeded in six-well plates and transfected with pcDNA3.1-ATG2B and siRNA-ATG2B, the pcDNA3.1 and siRNA empty vectors were served as a negative control (NC). Subsequently, the cells were collected for transfection efficiency analysis and examined for autophagy and gene expression 48 h after transfection.

## 2.10 | Measurement of cell autophagy

Ovarian GCs in good growth condition were digested and counted and added to a 24-well plate lined with cell crawlers at 500 μL/well ($1.5 \times 10^5$ cells). Cell autophagy levels were detected using adenovirus-harboring tandem fluorescent mRFP-GFP-LC3 (Hanbio), according to the manufacturer's instructions. Cells cultured for 24–48 h were removed from the crawl to slides, sealed with a put-quenched sealer, and observed for fluorescence at 587 nm (red light) and 488 nm (green light) using confocal microscopy.

## 2.11 | MLT reagent

MLT (M5250-1G; Sigma-Aldrich) was dissolved in dimethyl sulfoxide and stored at −20°C. Before use, a stock solution of MLT in PBS at the desired concentrations was directly cultured with the indicated GCs.

## 2.12 | Cell counting Kit-8 (CCK-8) assay

Sheep GCs in the added medium were seeded in 96-well cell culture plates. When the cell density reached an appropriate level, the cells were transfected. Cell proliferation was detected after transfection for 0, 6, 12, 24, and 48 h according to the instructions of the CCK-8 Kit (Vazyme), and absorbance was measured at 450 nm using a multi-mode micropore detection system (EnSpire, Perkin Elmer).

## 2.13 | Ethynyldeoxyuridine (EdU) assay

The proliferation of sheep GCs was quantified using an EdU kit according to the manufacturer's protocol (Beyotime). EdU-stained cells were observed and quantified using fluorescence microscopy. Three regions were randomly selected for quantification and statistical analysis.

## 2.14 | Statistical analysis

Data were statistically analyzed using SPSS 20 statistical software, and the means of three replicates were evaluated and shown as the mean ± standard error (SE). The results of Western blot analysis and cell autophagy were analyzed using the ImageJ software. Histograms were constructed using Excel and GraphPad Prism. Significance was determined using Duncan's multiple range test and is presented as $^*p < .05$ and $^{**}p < .01$.

# 3 | RESULTS

## 3.1 | TaqMan typing results of sheep GCs with different *Fec*$^B$ genotypes

To obtain sheep GCs with different *Fec*$^B$ genotypes, the genotypes of the sheep follicular GCs were detected using the TaqMan probe method (Figure 1B). The results showed that the ovarian tissue was generally larger in *Fec*$^B$ ++ individuals than that in *Fec*$^B$ BB individuals, with no significant difference ($p > .05$) (Figure 1A). The purity of the obtained GCs was assessed using the FSHR immunofluorescence assay. The cell purity was >90% (Figure 1C) and could be used for subsequent analyses.

## 3.2 | Viability and hormone secretion assay of GCs with different *Fec*$^B$ genotypes

To reveal the differences between GCs with different *Fec*$^B$ genotypes, the viability and secreted hormones of GCs with both genotypes were examined. CCK-8 and EdU assays showed that the proliferation rate of GCs in *Fec*$^B$ BB genotype was significantly higher than that in *Fec*$^B$ ++ genotype (Figure 2A and 1B). ELISA results showed that $E_2$ and PORG levels were significantly higher in both GCs and culture medium of the *Fec*$^B$ BB genotype than those in the *Fec*$^B$ ++ genotype ($p < .05$) (Figure 2C). The vacuole formation rate in the *Fec*$^B$ BB genotype GCs was lower than that in *Fec*$^B$ ++ genotype (Figure 2D). These results indicate that GC viability and hormone secretory capacity were significantly higher in the *Fec*$^B$ BB genotype than those in the *Fec*$^B$ ++ genotype.

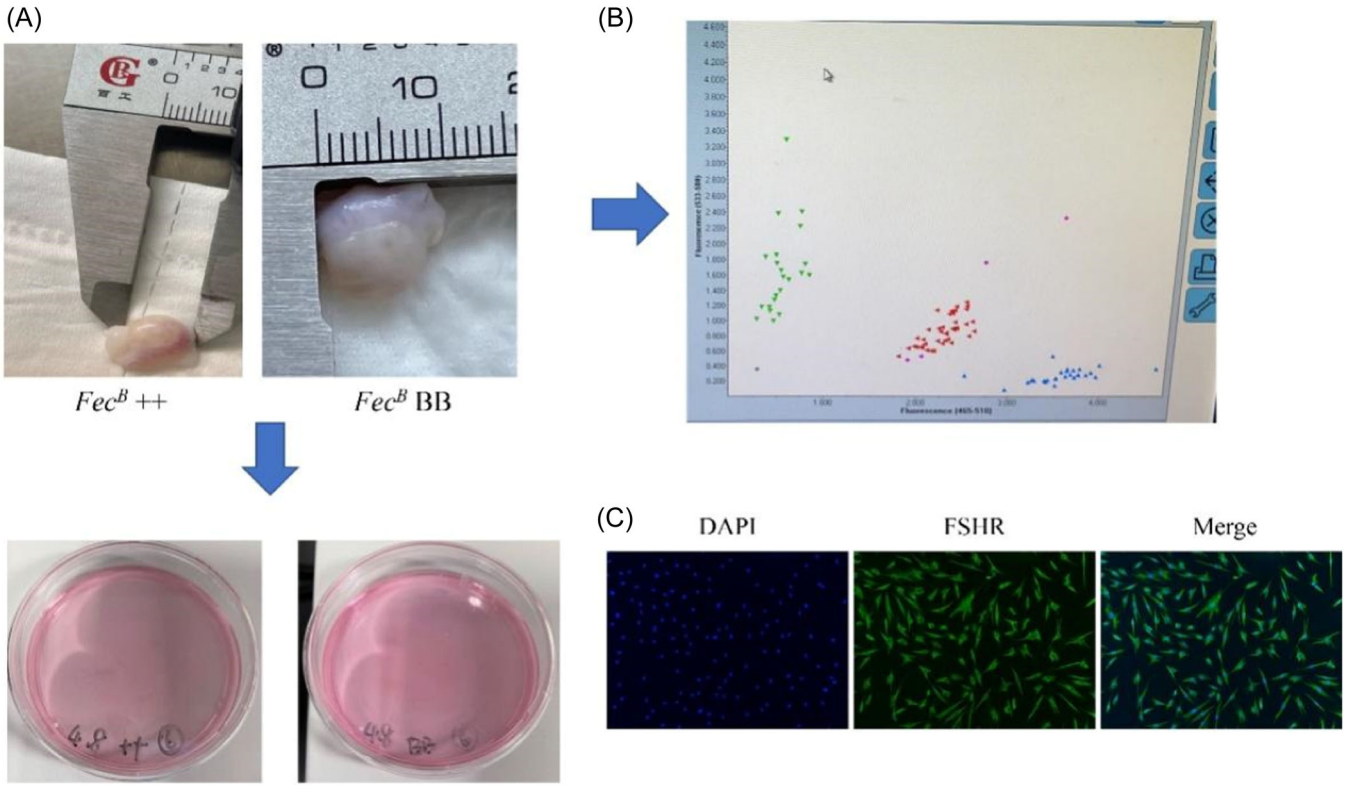


FIGURE 1 Isolation and identification of sheep granulosa cells with different $Fec^B$ genotypes (A) Ovarian tissue of different $Fec^B$ genotypes. (B) Results of TaqMan typing. (C) Results of FSHR immunofluorescence assay. Note: The green point represents the granulosa cells with $Fec^B$ BB genotype, blue point represents the granulosa cells with $Fec^B$ ++ genotype, and purple point represents the granulosa cells with $Fec^B$ + B genotype.

### 3.3 | Differential analysis of cell proliferation, apoptosis, and autophagy in GCs with different $Fec^B$ genotypes

To investigate the differences in cell proliferation, apoptosis, and autophagy between $Fec^B$ BB and ++ GCs, their markers were examined. The results showed that the levels of the cellular autophagy markers ATG7 and LC3 were significantly higher in $Fec^B$ BB genotype than in those of the $Fec^B$ ++ genotype ($p < .05$) (Figure 3A,B). However, cell proliferation and apoptosis did not differ significantly between GCs with $Fec^B$ BB and ++ genotypes ($p > .05$) (Figure 3C,D). The results of ROS and autophagosome detection showed that the levels of ROS and autophagosomes in $Fec^B$ BB genotype were significantly higher than those in $Fec^B$ ++ genotype ($p < .05$) (Figure 3E,F).

### 3.4 | *ATG2B* promoted sheep GC autophagy

To investigate the relationship between *ATG2B* and autophagy in GCs, its expression was analyzed in GCs with different $Fec^B$ genotypes. RT-qPCR and western blot analysis results showed that *ATG2B* expression was significantly higher in GCs with $Fec^B$ BB genotype than that in GCs with the $Fec^B$ ++ genotype ($p < .05$) (Figure 4A,B). These results are consistent with the single-cell sequencing results. To further elucidate the molecular mechanism underlying *ATG2B* regulation of GC autophagy, we constructed overexpression and interference plasmids. Transfection of GCs showed that both the overexpression and interference plasmids functioned properly (Figure 4C). The GC vacuole rate assay showed that the vacuolation rate in GCs with both $Fec^B$ genotypes after overexpression of *ATG2B* was significantly lower than that in the interfering group, especially in the $Fec^B$ ++ genotype (Figure 4D). ROS and mitochondria (mit) levels were significantly lower in the overexpression ATG2B group than those in the NC group for both $Fec^B$ genotypes. The reverse was true after inhibition, which was especially evident in the $Fec^B$ ++ genotype (Figure 4E,F). Autophagosome detection showed that GC autophagy was significantly increased in both $Fec^B$ genotypes after *ATG2B* overexpression compared to the NC group, and the reverse was true after inhibition, which was especially evident in the

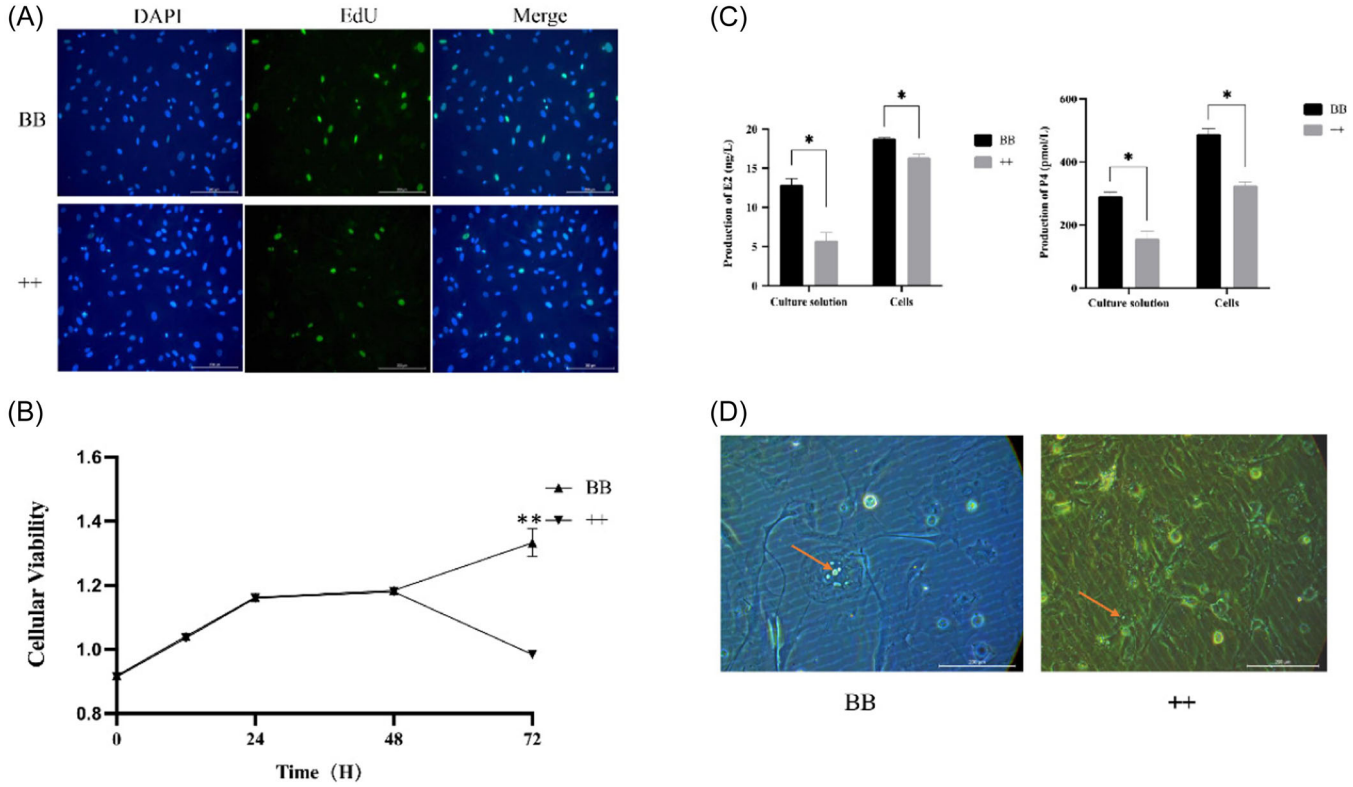


FIGURE 2 Detection of granulosa cell viability and hormone secretory capacity of different $Fec^B$ genotypes (A) EdU assay of granulosa cells with different $Fec^B$ genotypes. (B) Result of CCK-8 assays of granulosa cells with different $Fec^B$ genotypes. (C) Results of ELISA. (D) Vacuole rate detection in granulosa cells with different $Fec^B$ genotypes. *$p < .05$; *$p < .01$.

$Fec^B$ ++ genotype (Figure 4G,H). These results indicate that *ATG2B* promoted GC autophagy and was significantly more functional in GCs with $Fec^B$ ++ genotype than that in GCs with $Fec^B$ BB genotype.

## 3.5 | Effect of MLT on the viability of GCs with different $Fec^B$ genotypes

To further clarify the influence of MLT on GCs, the expression and secretion levels of MLT and the expression of MLT receptor 1A (MTNR1A) in GCs of small-tailed Han sheep with different genotypes of $Fec^B$ were detected. The results showed that secretion of MLT in the GCs of small-tailed Han sheep with different genotypes of $Fec^B$ was low and not significantly different ($p > .05$). There was also no significant different in the expression of *MTNR1A* in GCs of small-tailed Han sheep with different $Fec^B$ genotypes ($p > .05$) (Supporting Information: Figure S1A). Therefore, the secretion and expression of MLT in the GCs do not affect the effect of MLT addition in vitro. To investigate the role of MLT in GC autophagy, its effect on GC viability was tested by adding MLT. The results showed that the expression of cell proliferation factors and secretion of $E_2$ and PROG reached their highest levels after the addition of MLT at concentrations of $10^{-11}$ mol/L in vitro (Figure 5A,B). The expression of *ATG2B* was significantly increased in GCs with different $Fec^B$ genotypes with a constant pattern (Figure 5C). EdU and CCK-8 assays showed that GC proliferation significantly increased after the addition of MLT, which was especially significant in GCs with $Fec^B$ ++ genotype (Figure 5D,E).

## 3.6 | Effect of MLT on autophagy in GCs with different $Fec^B$ genotypes

To further investigate the effect of MLT on autophagy in GCs with different $Fec^B$ genotypes, GC autophagy levels were examined after the addition of MLT. The results showed that the vacuolation rate significantly decreased in both $Fec^B$ genotypes after the addition of MLT, which was especially significant in the GCs with the $Fec^B$ ++ genotype (Figure 6A). ROS and mit levels decreased in the GCs with both $Fec^B$ genotypes, which was especially evident in the $Fec^B$ ++ genotype ($p < .05$) (Figure 6B). The addition of MLT significantly reduced autophagosome levels in GCs

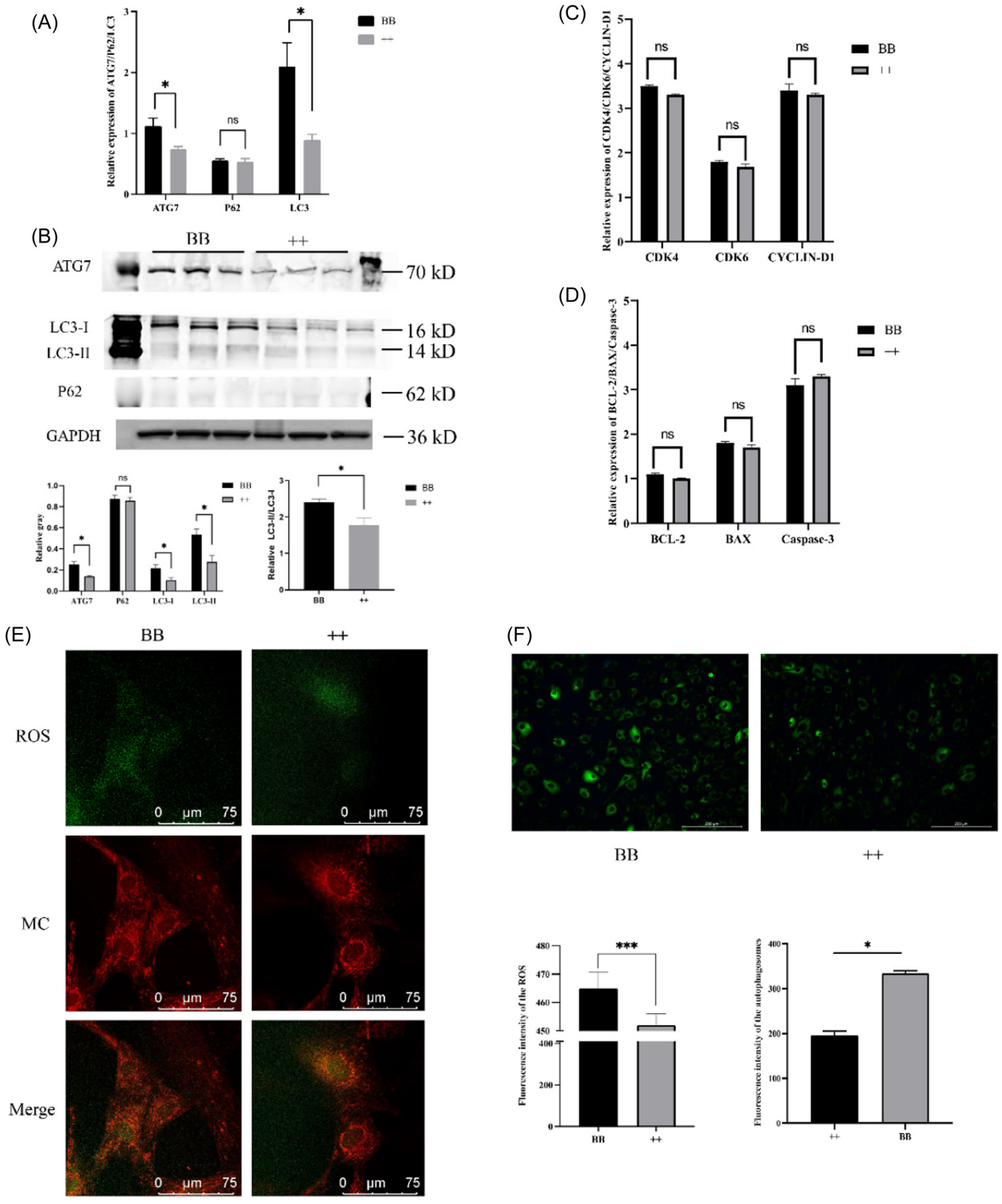


**FIGURE 3** Autophagy level of $Fec^B$ BB genotype granulosa cells is higher than that of $Fec^B$ ++ genotype. (A) mRNA expression of *ATG7, P62*, and *LC3*. (B) Protein expression of ATG7, P62, LC3-I, and LC3-II, and their gray value analysis. (C) mRNA expression of cell proliferation markers (*CDK4, CDK6*, and Cyclin D1). (D) mRNA expression of cell apoptosis markers (*BCL-2, BAX*, and Caspase-3). (E) ROS level detection in granulosa cells with different $Fec^B$ genotypes. (F) Autophagosome detection in granulosa cells with different $Fec^B$ genotypes. $^*p < .05$. ns, no significant difference.

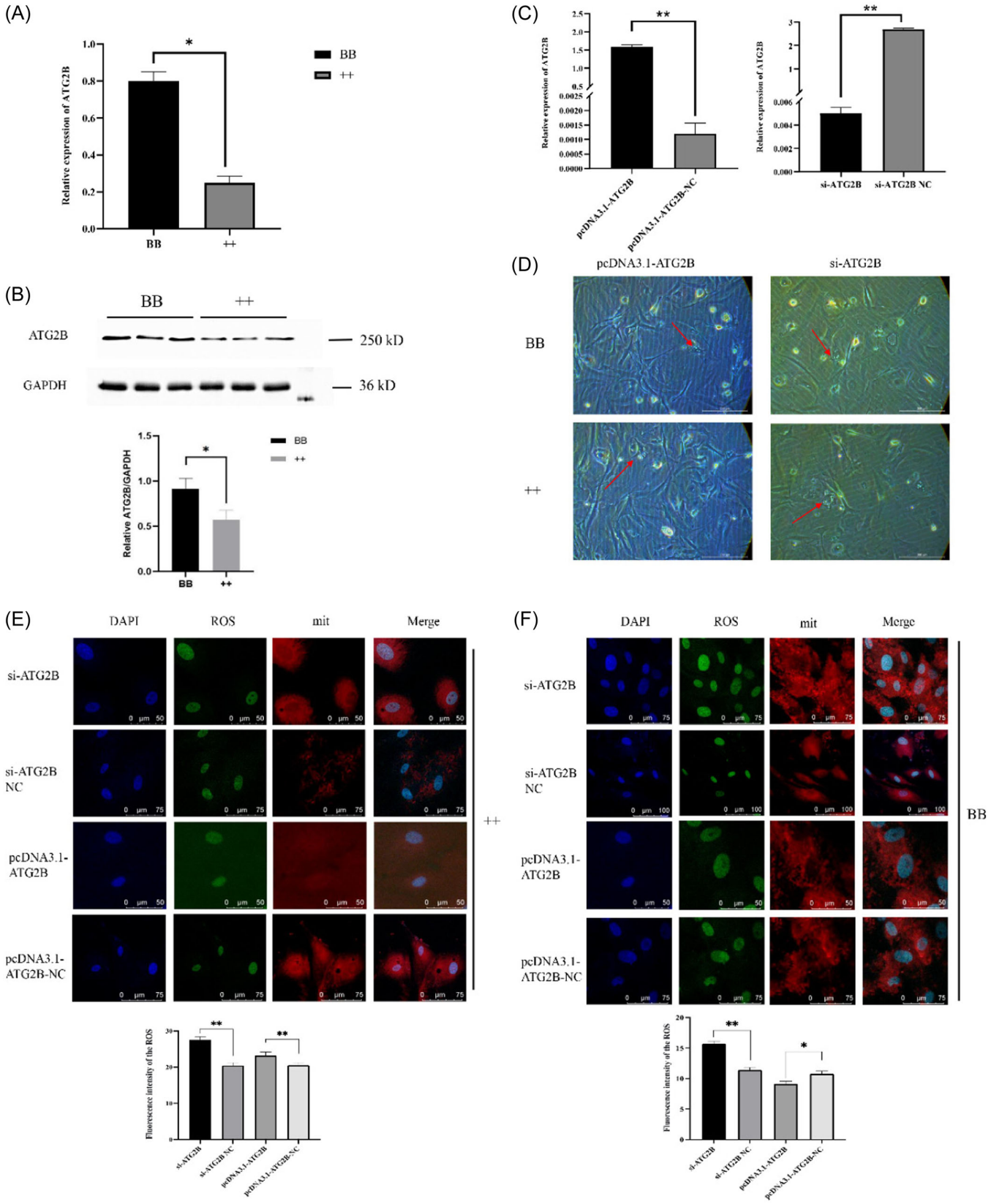


**FIGURE 4** *ATG2B* promotes granulosa cell autophagy. (A) Relative expression of *ATG2B* in different $Fec^B$ genotypes. (B) Western blot and gray value analysis of ATG2B in different $Fec^B$ genotypes. (C) Relative expression of *ATG2B* after overexpression or interference of *ATG2B*. (D) Detection of vacuole rate in granulosa cells with different $Fec^B$ genotypes after overexpression or interference of *ATG2B*. (E) ROS and mit level detection after overexpression or interference of *ATG2B* in granulosa cells with $Fec^B$ ++ genotype. (F) ROS and mit level detection after overexpression or interference of *ATG2B* in granulosa cells with $Fec^B$ BB genotype. (G) Autophagosome detection after overexpression or interference of *ATG2B* in granulosa cells with $Fec^B$ ++ genotype. (H) Detection after overexpression or interference of *ATG2B* in granulosa cells with $Fec^B$ BB genotype. $^*p < .05$; $^{**}p < .01$.

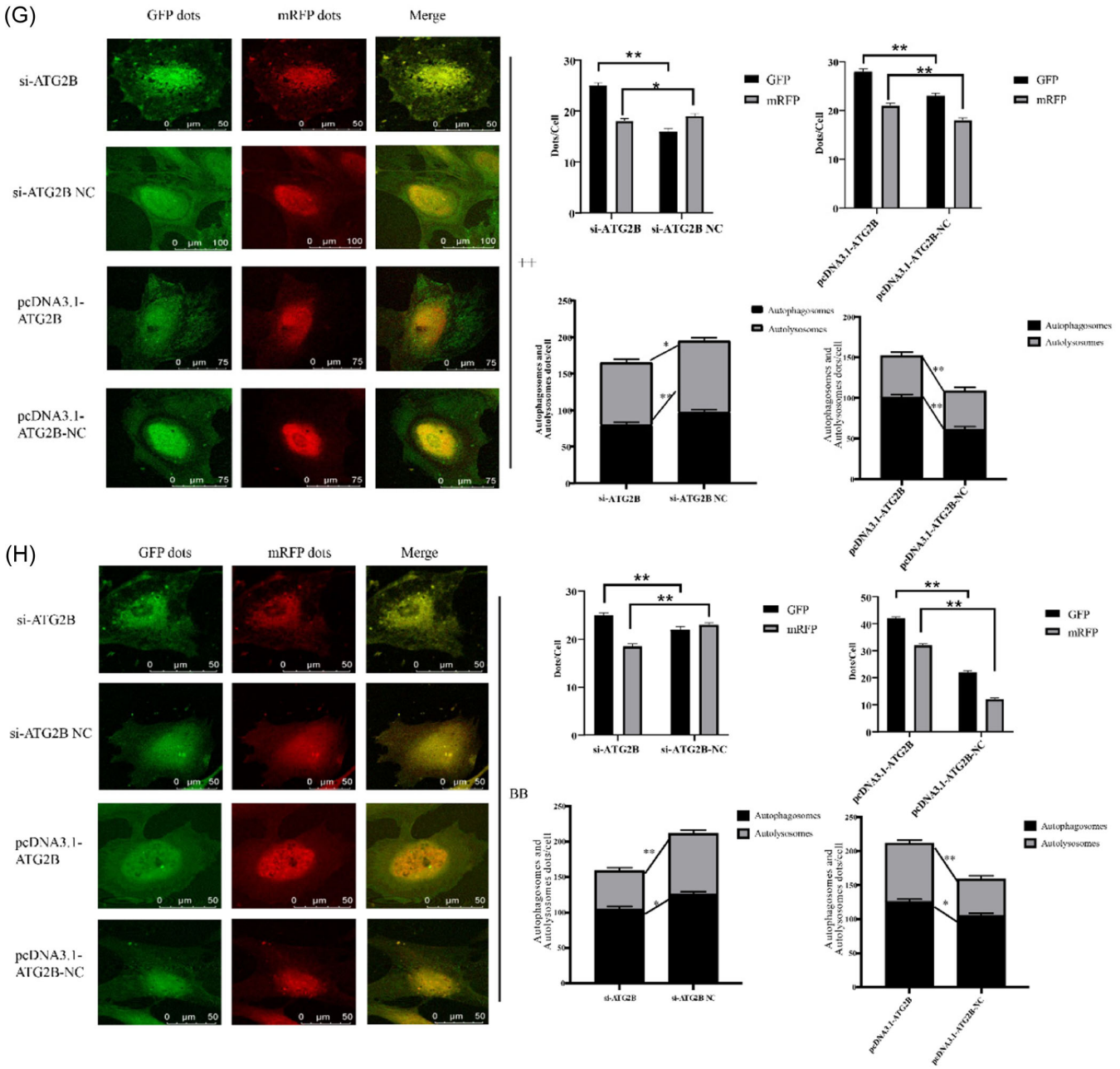


**FIGURE 4** (Continued)

with both $Fec^B$ genotypes, suggesting that MLT inhibited autophagy and protected the cells by reducing ROS and mit levels ($p < .05$) (Figure 6C).

## 3.7 | MLT along with *ATG2B* regulated ROS-dependent autophagy induced by GCs with different $Fec^B$ genotypes

To determine whether the *ATG2B* may regulate MLT levels in GCs, the expression and secretion of MLT in GCs of small-tailed Han sheep with different $Fec^B$ genotypes were detected after inhibition of *ATG2B*. The results showed that the expression and secretion of MLT in GCs of small-tailed Han sheep with different $Fec^B$ genotypes was decreased than that of $Fec^B$++ genotype after overexpression or inhibition with *ATG2B* but the difference was not significant ($p > .05$), and the expression of *MTNR1A* was also not significantly different in GCs of different $Fec^B$ genotypes (Supporting Information: Figure S1B). To investigate the role of MLT in *ATG2B* regulation of autophagy in GCs with different $Fec^B$ genotypes, MLT was added after the inhibition of *ATG2B* expression in GCs. The inhibition of *ATG2B* expression

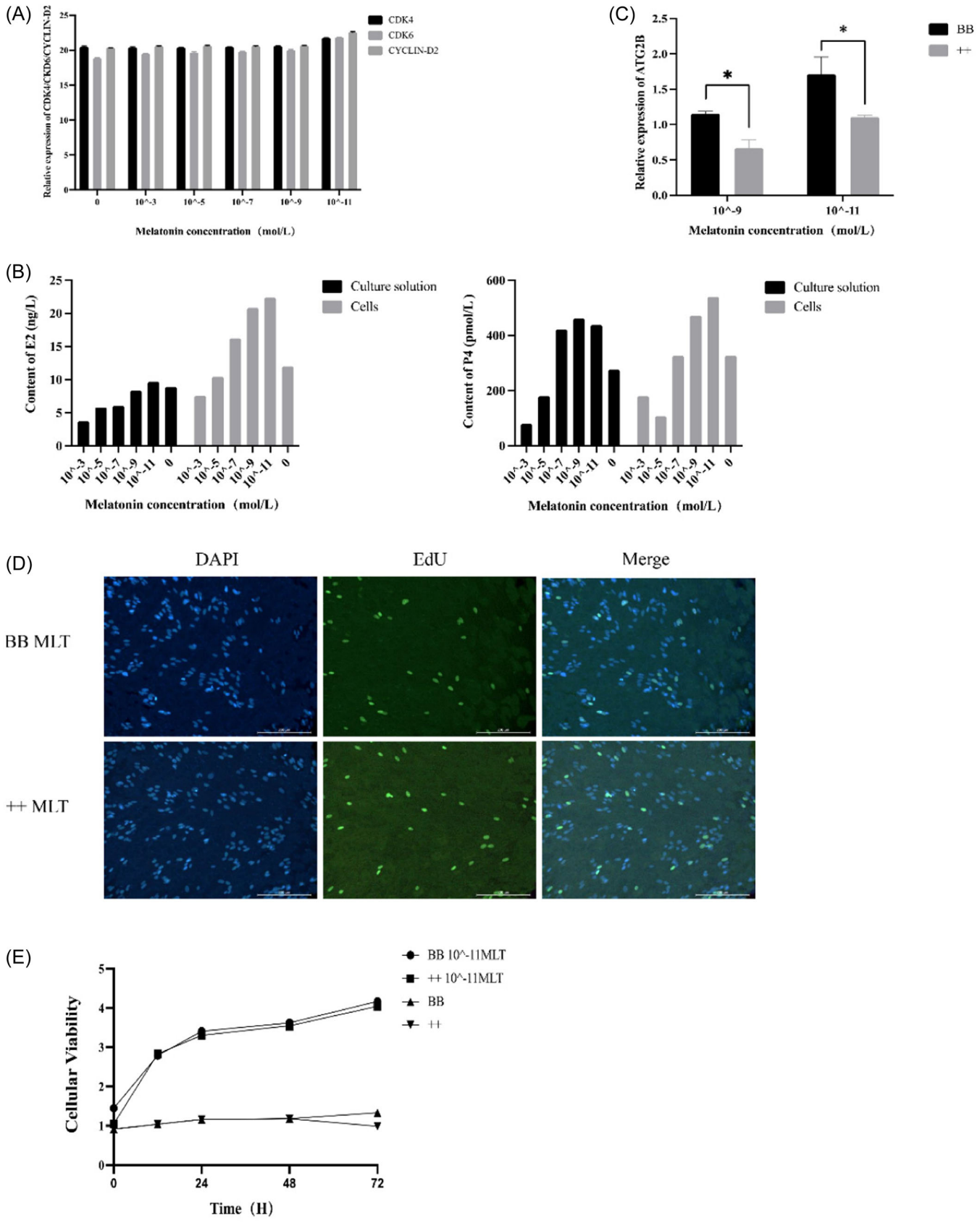


FIGURE 5 (See caption on next page)

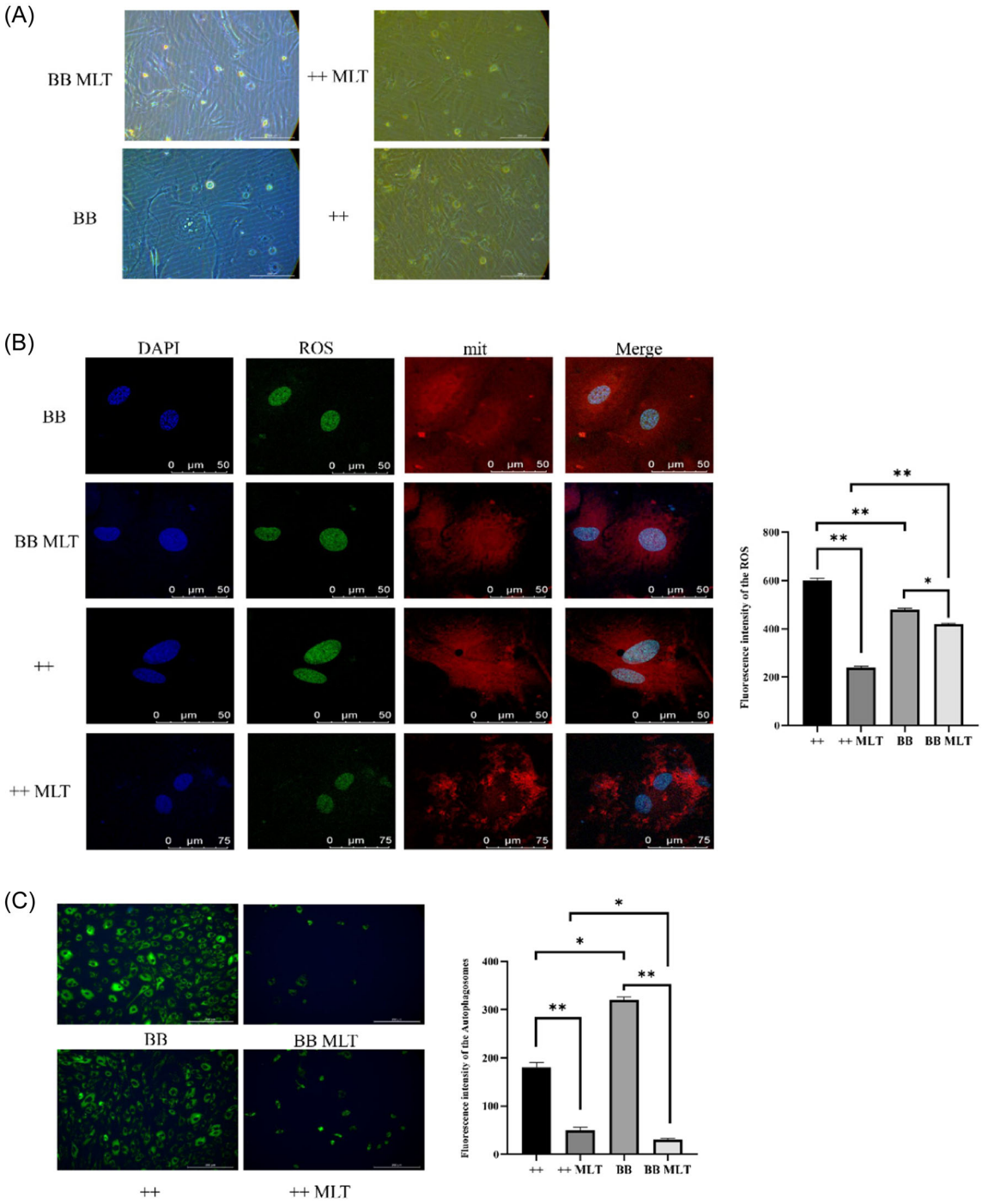


**FIGURE 6** In vitro addition of melatonin inhibits autophagy in granulosa cells of different $Fec^B$ genotypes (A) Detection of air bubble ratio in granulosa cells with different $Fec^B$ genotypes after MLT addition. (B) ROS and mit level detection in granulosa cells with different $Fec^B$ genotypes after MLT addition. (C) Autophagosome level detection in granulosa cells with different $Fec^B$ genotypes after MLT addition. $^*p < .05$; $^{**}p < .01$. ROS, reactive oxygen species.

**FIGURE 5** In vitro addition of melatonin promotes granulosa cell activity. (A) Expression of different concentrations of melatonin granule cell proliferation factor. (B) Detection of $E_2$ and PROG secretion in melatonin granule cells at different concentrations. (C) Relative expression of *ATG2B* in granulosa cells with different $Fec^B$ genotypes after melatonin addition. (D) EdU detection after melatonin addition in granulosa cells with different $Fec^B$ genotypes. (E) CCK-8 assay after melatonin addition in granulosa cells with different $Fec^B$ genotypes. $^*p < .05$; $^{**}p < .01$.

resulted in a significant decrease in the expression of cellular autophagy marker factors in GCs with both $Fec^B$ genotypes (Figure 7A,B). ROS and mit levels were significantly decreased in the MLT-treated group compared to those in the NC group in GCs with both $Fec^B$ genotypes that inhibited *ATG2B* expression (Figure 7C). The autophagosome levels showed the same trend (Figure 7D). These findings suggest that MLT was able to reduce ROS and mit generated after *ATG2B* interference in a manner that was not mediated by cellular autophagy. MLT can act synergistically with *ATG2B* to protect sheep GCs, especially $Fec^B$ ++ GCs.

## 4 | DISCUSSION

*FecB* mutation has emerged as a candidate for marker-assisted selection for breeding sheep with high prolificacy.[27,28] However, the molecular mechanisms underlying differences in ovulation numbers in ewes with different $Fec^B$ genotypes require further investigation. Physiological analysis has revealed that the product of $Fec^B$ affects ovarian activity, especially in ovarian follicles, where the gene product directly or indirectly induces premature ovarian follicular maturation.[29] Ewes carrying the $Fec^B$ BB genotype were partially inactivated in the Booroola strain of Australian Merino sheep compared to non-carriers with bone morphogenetic receptor type 1 B (BMPR-1B), resulting in advanced GC differentiation and maturation of ovulating follicles.[3] PCR-RFLP and PCR-SSCP detection techniques for the early breeding of multi-lamb sheep were established based on the effects of $Fec^B$ mutation. More than 80,000 sheep were typed using these techniques, and two new multi-lamb breeds were bred. The results of the present study reaffirm the role of the $Fec^B$ gene in promoting ovulation. In the present study, sheep GCs with both $Fec^B$ BB and ++ genotypes were obtained using the TaqMan probe method. A research foundation was provided for the synergistic action of MLT and *ATG2B* on GCs with different genotypes of $Fec^B$ to protect GCs by regulating autophagy levels, thereby promoting hormone secretion.

Cellular autophagy, an intracellular degradation system for recycling damaged organelles and removing aggregated proteins via lysosomes, is essential for maintaining cellular homeostasis.[30] In response to external stress conditions, such as nutritional deficiency and hypoxia, autophagy promotes cell survival by degrading cellular components to release energy and eliminate defective or damaged organelles and proteins.[31] Increased ROS levels are an important trigger for cell autophagy.[32] The current findings showed that ROS levels in GCs with $Fec^B$ BB genotype were significantly higher than those in GCs with $Fec^B$ ++ genotype and that autophagy levels were higher in GCs with $Fec^B$ BB genotype than those in GCs with $Fec^B$ ++ genotype.

ROS are highly reactive small molecules that oxidize proteins, lipids, and DNA; act as signaling molecules to regulate the activity of oxidized targets; and play an important role in autophagy.[33] There are several direct and indirect responses to elevated ROS levels. First, increased ROS levels promote the expression of the autophagy-associated genes *Beclin-1, ATG4, and P62*.[34,35] Thus, mitochondrial autophagy occurring in cells reduces the effects of ROS and protects cells by providing them with energy.[36] In our previous study on $Fec^B$ BB and ++ GC single-cell sequencing (unpublished data), we found that *ATG2B* was significantly more highly expressed in the $Fec^B$ BB genotype GCs than that in the $Fec^B$ ++ genotype. *ATG2B* is significantly associated with cellular autophagy.[37] The role of this gene in GC autophagy was explored in the present study. In the present study, overexpression or inhibition of *ATG2B* revealed that GC autophagy was significantly increased in both $Fec^B$ genotypes, especially in $Fec^B$ ++ GCs. Therefore, the high expression of *ATG2B* in GCs with different $Fec^B$ genotypes promoted ROS-induced GC autophagy, which provided protection and energy supply to GCs, and the autophagy caused by the difference in *ATG2B* expression level might be the reason for the difference in ovulation number of individuals with different $Fec^B$ genotypes. These findings suggest that in sheep without the $Fec^B$ mutation, the ability to increase GC autophagy has the potential to increase GC resistance to the external environment, thereby promoting GC proliferation and affecting lambing numbers.

Second, MLT (5-methoxy-N-acetyltryptamine) is an endogenous hormone released by the pineal gland that regulates mitochondria-related functions and strengthens the antioxidant defense system by scavenging toxic free radicals.[38,39] In the present study, the addition of MLT at a concentration of 10^−11 mol/L significantly increased GC activity, sex hormone secretion, and expression of *ATG2B* in different $Fec^B$ genotypes. The addition of MLT to GCs with different $Fec^B$ genotypes significantly decreased ROS and GC autophagy levels. This result suggests that MLT protects cells by directly reducing ROS levels. To further reveal the roles of MLT and *ATG2B* in GCs with different $Fec^B$ genotypes, the addition of MLT to GCs with inhibited *ATG2B* expression resulted in a significant reduction in ROS and autophagy levels, which was particularly significant in $Fec^B$ ++ GCs. These results suggest that MLT and *ATG2B* act synergistically on ROS production in GCs, where both MLT and *ATG2B* reduced ROS levels, thus protecting cells by promoting cellular autophagy, especially in

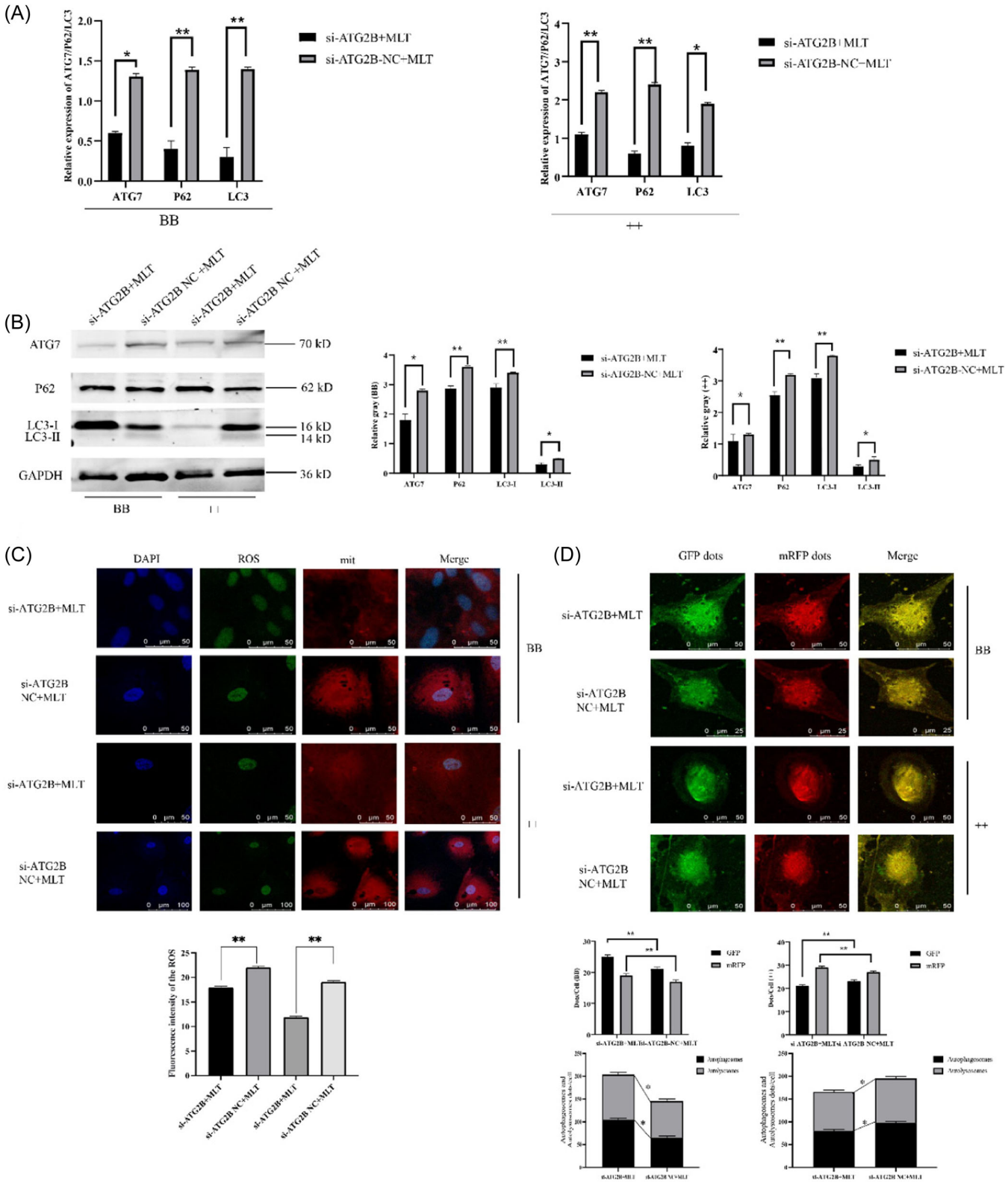


**FIGURE 7** MLT acts synergistically with ATG2B to regulate granulosa cell autophagy (A) Relative expression of cell autophagy markers after addition of MLT in granulosa cells with both $Fec^B$ genotypes that inhibited *ATG2B* expression. (B) Western blot results of cell autophagy markers after addition of MLT in granulosa cells with both $Fec^B$ genotypes that inhibited *ATG2B* expression. (C) ROS and mit detection after addition of MLT in granulosa cells with both $Fec^B$ genotypes that inhibited *ATG2B* expression. (D) Detection of autophagosome levels after addition of MLT in the granulosa cells with both $Fec^B$ genotypes that inhibited *ATG2B* expression. $^*p < .05$; $^{**}p < .01$. MLT, Melatonin.

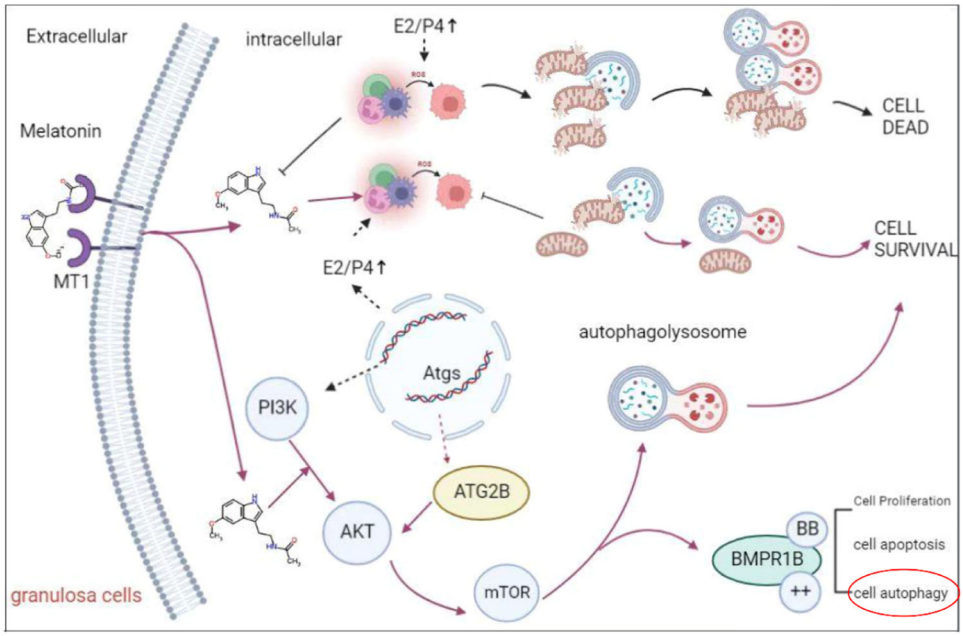


**FIGURE 8** Schematic diagram showing that melatonin can act along with *ATG2B* to regulate autophagy in granulosa cells with different $Fec^B$ genotypes.

$Fec^B$ ++ GCs. Therefore, the addition of MLT along with the overexpression of *ATG2B* in the breeding system of small-tailed Han sheep without $Fec^B$ mutation has the potential to protect GCs by increasing the level of cellular autophagy, reducing GC autophagy, and providing good survival conditions for oocyte maturation, thus promoting ovulation.

In conclusion, our data show for the first time that there is a significant difference in the level of autophagy in GCs of individuals with the $Fec^B$ BB and $Fec^B$ ++ genotypes and that the regulation of autophagy by the differentially expressed gene *ATG2B* may be important in influencing hormonal secretion in GCs with different $Fec^B$ genotypes. MLT can act together with *ATG2B* to regulate autophagy in GCs with different $Fec^B$ genotypes (Figure 8), and this pathway of action provides a novel perspective to reveal the molecular mechanisms underlying the differences in ovulation number in individuals with different $Fec^B$ genotypes.

## AUTHOR CONTRIBUTIONS

Ming-Xing Chu guided research subject selection, experimental design, and manuscript writing and revision. Yu-Fang Liu designed and performed the experiments, and wrote the original manuscript. Zi-Yi Liu analyzed and visualized the data and wrote the original manuscript. Yu-Fang Liu, Zi-Yi Liu, Peng Wang, Wen-Tao Li, Xiang-Yu Wang, Ran Di, and Xiao-Yun He performed the experiments. Ming-Xing Chu critically reviewed the manuscript. All authors have approved the manuscript and its version for publication.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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