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# MiR-98 regulates proliferation, apoptosis, and estrogen secretion in goat ovarian granulosa cells by targeting COL1A1

**Abstract**: Ovarian granulosa cells (OGCs) provide nutrients to oocytes by expressing and secreting follicle-stimulating hormone receptors and regulating the entire follicular development, oocyte maturation, and ovulation process. Collagen type I α1 chain (COLA1 encoded by the gene *COL1A1*) and miRNAs have been found to play important roles in the proliferation, migration, and apoptosis of goat OGCs. However, the specific miRNAs involved in the regulation of these processes remain unknown. This study aimed to examine the effects of miRNA-targeted regulation of *COL1A1* on proliferation, migration, apoptosis, and hormone secretion in goat OGCs. Computational biology was employed to predict the miRNAs that target and regulate *COL1A1* expression in goat OGCs. The expression of candidate miRNAs in the ovary was detected by fluorescence quantitative polymerase chain reaction and identified the miRNAs regulating *COL1A1* expression. Next, the dual-luciferase reporter gene assay was performed to test the targeting and regulating effects between miRNAs and *COL1A1*. Finally, the CCK-8, cell scoring, flow cytometry, and other assays were used to test the targeting and regulatory functions of *COL1A1*. The effects of miRNAs on cell proliferation, cell migration, cell cycle, apoptosis, and hormone secretion of OGCs were assessed by cell scratch, flow cytometry, western blotting, quantitative real-time-polymerase chain reaction, and ELISA to detect. Computational biology was employed and miR-29d-3p, miR-31, miR-98, miR-218, and miR-133 were predicted to target and regulate the *COL1A1*. Compared with the other four miRNAs, the expression of miR-98 was extremely significantly reduced in the ovarian tissues of ewes with consecutive multi lambs in Guizhou Black Goats. MiR-98 targets and inhibits the expression of the *COL1A1*. Functionality study of miR-98 revealed that miR-98 overexpression in the OGCs significantly inhibited cell proliferation and cell migration and promoted early apoptosis. Overexpression of miR-98 not only blocked the normal cell cycle, cell proliferation, and cell migration and promoted early apoptosis but also inhibited the secretion of E2 by OGCs. This study revealed the regulatory role of miR-98 by targeting *COL1A1* in the proliferation, migration, apoptosis, and hormone secretion of OGCs in Guizhou Black Goats. This study lays the foundation for further investigation of the effects of non-coding RNAs on the reproductive ability of Guizhou black goats.

**Keywords:** miR-98; *COL1A1*; ovarian granulosa cells; cell proliferation; apoptosis

## 1. Introduction

The follicle is the core functional unit of the female mammalian reproductive system (1). It begins with the activation of primordial follicles, which are small, inactive follicles in the ovary (2). Once activated, they develop into primary follicles, where granulosa cells (follicular cells) proliferate and form multiple layers around the oocyte (3). The process continues as the follicle grows, eventually becoming a mature, pre-ovulatory (Graafian) follicle ready for ovulation (4).The development of follicles and atresia is regulated by a combination of factors, including hormones, growth factors, and specific genes (5). During the maturation of follicles, ovarian granulosa cells (OGCs) are crucial (6). OGCs are responsible for regulating the internal environment of the follicle and directly influence follicular maturation and selection through their own proliferative and secretory activities (7). OGCs determine follicle survival or atresia by finely regulating apoptosis, which is critical for ovarian health and fertility (8, 9). *COL1A1*, a major component of the extracellular matrix, serves as the structural basis for a variety of tissues and plays a role in disease progression, ovarian development, and ovulation disorders (10-14). Studies on human diseases have unequivocally demonstrated a close association of *COL1A1* with a multitude of pathological states, especially in osteoporosis, tumor invasion and metastasis, and diseases associated with the remodeling of extracellular matrix (ECM) (14-18) .In various tissues, COL1A1 supports cell adhesion, proliferation, and differentiation, playing a fundamental role in wound healing and tissue remodeling (19). In follicular cells, COL1A1 is anticipated to be involved in follicular development by influencing the extracellular matrix's composition and stability, which is crucial for supporting cell proliferation and oocyte maturation (20). In the field of research, especially on the fertility in domestic animals, *COL1A1* is closely associated with ovarian angiogenesis and development, remodeling of the embryonic mesenchyme, ovarian atresia, and polycystic ovary syndrome (PCOS) (21-23). In cattle, *COL1A1* regulates bovine fetal ovarian development and interstitial remodeling, and its expression level is associated with morphological changes in the structural organization of the ovary (24). Our group found that *COL1A1* has a dose-effect relationship with the reproductive rate of Guizhou black goats. Furthermore, the ovarian tissues of ewes in the continuous multiple lambing group exhibit significantly higher expression of the *COL1A1* gene than ewes in the continuous single lambing group. Our findings show that overexpression of *COL1A1* promotes the expression of lambing-associated genes and OGCs proliferation and migration. Conversely, interference with the *COL1A1* gene expression has the opposite effect. MiRNAs are highly conserved non-coding RNA (nc-RNA) molecules of 18-25

nucleotides length (25) . They initiate mRNA degradation or inhibit their translation by pairing with the 3' non-coding region (UTR) of target genes, stopping post-transcriptional gene expression and affecting a number of key physiological processes, including cell growth, development, proliferation, and apoptosis(26) . MiRNAs also play specific roles in reproductive functions in female mammals, including the development of the ovaries, follicular maturation, OGC proliferation, apoptosis, and secretion of estrogen and progesterone (15, 20, 27-29). However, the molecular mechanism through which miRNAs regulate *COL1A1* in goat OGCs to affect cell proliferation and apoptosis is still not clear. Besides,miR-98 widely involved in the physiological processes of cell growth, apoptosis, migration, and invasion in various cancer cells (30).In the current study, We found that miR-98, as one of the candidate miRNAs regulating the *COL1A1* gene expression, was significantly down-regulated in the ovaries of the multiple lambing group ewes, contrary to *COL1A1* gene expression in the above-mentioned tissues. Therefore, based on the previous research, this study used *COL1A1* as the research object, screened miRNAs targeting *COL1A1* by bioinformatics analysis software were screened and the targeting and regulation effect of miRNAs on *COL1A1* were verified by dual-luciferase reporter system. Further, the effects of miRNAs targeting *COL1A1* on proliferation and migration, cell cycle, apoptosis, gene expression, and hormone secretion of goat OGCs were determined, and the regulatory effects and molecular mechanisms associated with miRNAs targeting *COL1A1* on OGCs growth, development, and hormone synthesis were analyzed. The study findings provide a basis to further explore the NC-RNAs along with the key candidate genes to regulate the fertility in goats, and pave the way for further research into the molecular mechanism involved in the role of NC-RNAs and key candidate genes in the regulation of the reproductive ability of goats.



## 2. Materials

### 2.1 Tissues and cells

The ovary tissue samples used in this study were procured from the Maiping Black Goat Breeding Base, Guizhou Provincial Animal Husbandry and Veterinary Research Institute,choose a 4-year-old ewe with two consecutive litters of twin lambs or single lambs. The black goats were slaughtered and delivered back to the laboratory within 1 h, adhering strictly to the operational procedures and technical standards stipulated by the state. Goat OGCs were isolated from one side of the ovary and cultured, and the other side was used to isolate total RNA. For transgenerational preservation, cells of human embryonic kidney cell line 293T (HEK-293T) were preserved by the Key Laboratory of Genetic

Breeding and Reproduction, Plateau Mountain Animals of the Ministry of Education, China. The OGCs were identified by immunofluorescence and cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 complete culture medium containing fetal bovine serum (FBS; 10%) and penicillin-streptomycin (1%),allocate cryopreservation for later use while waiting for the cells to fill the flask. HEK-293T cells were cultured in a DMEM complete medium (containing 10% FBS and penicillin-streptomycin, 1%). Both types of cells were cultured in an incubator at 37 ℃ and 5% carbon dioxide concentration.

2.1.2 Isolated culture of ovarian granulosa cells

Fresh ovarian tissue was washed 3 times in 75% alcohol and sterile PBS buffer with bispecific antibodies, first in a Petri dish to trim off the excess tissue, then in a Petri dish containing DMEM/F12 complete medium (containing 10% fetal bovine serum and 1% cyan-streptomycin), using a 5 mL medical syringe to aspirate the medium, puncture the needle through multiple follicles on the surface of the ovary, and repeatedly pipette the follicular cavity to completely release the follicular fluid. The medium was then transferred to a 10 mL sterile centrifuge tube, centrifuged at 1 000 rpm for 10 minutes, the medium was discarded, and the appropriate amount of complete medium was added again to wash once. Finally, 4 mL of complete medium was added to make a single cell suspension, transferred to a cell culture flask and placed in a $CO_2$ incubator to continue culture, observed and performed the first fluid change every other day, and then the cells were changed every 48 h, and when the cells grew to about 80% of the cell culture flask, the cells could be frozen and stored at -80 °C.

2.2 Ethics statement

All experimental animals were handled following the principles of animal welfare ethics. The study was approved by the Experimental Animal Ethics Committee of Guizhou University (Ethics No. EAE-GZU-2023-T077, Guiyang; 13 March 2023)

2.3 Reagents

The kits used in this study were: Dual Luciferase Assay Kit, Promega; Plasmid Extraction Kit, Axygen; DNA Gel Recovery Kit, Axygen; 2×EsTaq MasterMix, KangWei; fetal bovine serum, opti-MEM high-glucose medium, DMEM/F-12 medium（ Gibco,ThermoFisherScientific,USA）; siRNA-Mate Transfection Reagents, Gibco. Other reagents, such as TAE buffer, PGL empty vector, restriction endonucleases *SalI* and *XhoI,* T4 DNA ligase, DL15000 DNA Marker, TRizol reagent, chloroform, isopropanol, qPCRMix, and diethylpyrocarbonate (DEPC) water were purchased from Guizhou Xibao

Biological Co. Additionally, the reverse transcription kit, DMEM, DMEM-F12, FBS, 0.25% trypsin, and opti-MEM were purchased from Thermo Fisher Scientific, USA.

**3. Methodology**

3.1 Screening of miRNAs targeting *COL1A1*

The miRNAs regulating the expression of the COL1A1 gene were predicted and screened by the online software TargetScan8.0 (www.targetsscan.org/) and miRSystem (http://mirsystem.cgm.ntu.edu.tw/). These tools utilize the targeting mechanism of miRNAs and mRNAs. Ruminants, such as goats and cattle have high homology in the 3'-UTR of the *COL1A1* gene; therefore, the top five highest-scoring miRNAs involved in the regulation of the *COL1A1* gene in goats were identified based on the bovine *COL1A1* gene (miR-29d-3p, miR-31, miR-98, miR-218, and miR-218).

3.2 Primer design and synthesis

The sequences of the genes involved in this study were derived from relevant, published goat gene sequences in NCBI (https: //www.ncbi.nlm.nih.gov/.) the gene numbers are mentioned in Table 1. The primers employed for reverse transcription and fluorescence qPCR were designed employing Primer 5.0. The sequences of the reverse transcription-specific neck-loop primers of the five miRNAs are mentioned in Table 2, and the primers used for real-time fluorescence qPCR are shown in Table 3. All the primers were synthesized by Shanghai Prime Biotech Co. The primers for real-time fluorescence qPCR are mentioned in Table 3, and all primers were synthesized by Shanghai Kengke Biological Co.

3.3 Real-time quantitative polymerase chain reaction

Total RNA was extracted from ovaries and OGCs of black goats in Guizhou Province using TRIzol reagent(BOYAO,Guang Zhou,China,). The mRNA was reverse transcribed using Thermo Scientific Revert Aid premix (containing DNase l), and the following spiking system: 2× RT buffer mix (1 μL), 20× enzyme mix (10 μL), total RNA (1 μL), RNase-free $dH_2O$ (8 μL). The miRNA was reverse transcribed employing Star Script III miRNA RT Kit (by Stem-loop) and the reaction system comprised: spiking system RNA sample (1 μL), miRNA/internal reference U6 Stem-loop RT primer (1 μL), 2 × StarScript III miRNA-L buffer (10 μL), StarScript III miRNA-L enzyme mix (1 μL), nuclease-free water (DEPC-treated; 7 μL). qRT-PCR was performed on a BioRad C1000 detection system. To estimate the expression of mRNA, the GAPDH gene was used as an internal reference, while for miRNA, the U6 gene was used as an internal reference. The expression levels of mRNAs and miRNAs in ovarian tissues and OGCs were analyzed using the $2^{-\Delta\Delta Ct}$ method.

3.4 Vector construction

From the region of interaction of miR-98 and the *COL1A1* gene, a fragment of nearly 70 bp in the 3'-UTR of the *COL1A1* gene was selected and introduced into the *Xho I* and *Sal I* cleavage sites of the vector to form the wild-type sequence (WT-COL1A1). Subsequently, based on the wild-type sequence, mutations were introduced to generate the mutant sequence (MT-COL1A1) (Figure 2-B). The above sequence was synthesized by Shanghai Prime Biological Co. and annealed to create the double strand. Then, the target DNA fragment was double digested using restriction endonucleases *Xho I* and *Sal I*, and purified and detected by agarose gel electrophoresis. Finally, the ligation of the pmirGLO vector and the target fragment was performed using T4 DNA ligase and JM109 receptor cells were transformed with these constructs for further culturing and characterization. Finally, the plasmid was sequenced to confirm that the correct construct was selected.

3.5 Dual luciferase reporter test

The pGL-WT-COL1A1/3'-UTR wild-type, successfully constructed in the previous steps, was transfected along with the siRNA-mate transfection reagent (Gema,Shang Hai,China). The co-transfection of pGL-MT-COL1A1/3'-UTR mutant vector was performed, respectively, with miR-98 mimics、 NC mimics、mir-98 inhibit and NC inhibit, into 96-well plates, and was assayed using the Dualucif® Firefly&Renilla Assay Kit (URlandy, Suzhou, China). The firefly and renilla luminescence activities were determined employing a multifunctional enzyme marker (BioTek, Winooski, VT, USA) following the kit instructions. The results of this assay were expressed as the ratio of firefly to renilla luciferase activity per well.

3.6 Cell counting kit-8 test

The OGCs were inoculated in 96-well plates at 5 x $10^3$ cells/ well a day prior to transfection and cultured overnight. The next day, when the cell density reached about 50%, miR-98 and negative control (NC) were transfected using siRNA-mate. For each gradient, four time points (0 h, 24 h, 36 h, 48 h) were set up with six replicates, and incubated for 2 h after adding 10% CCK-8 reagent (URlandy, Suzhou, China) into each well. A multifunctional enzyme labeling apparatus (BioTek Winooski, VT, USA) was employed to determine the OD value at 450 nm for each time point, and the cell proliferation curve was prepared according to the OD value.

3.7 Cell scratch test

At the bottom of the 6-well plate, two parallel localization lines were drawn using a marker pen,

then $3 \times 10^5$ OGCs were evenly spread into each well and cultured overnight. The OGCs were cultured to 90%~95% confluence. The cells were scratched perpendicular to the localization lines using a 10.0 μL tip, and transfected with miR-98 and NC by rinsing with phosphate-buffered saline (PBS) 2~3 times. Then, the cells were examined by an inverted fluorescent microscope (Nikon C- SHG1) to visualize the migration area of the cells at 0 h and 24 h. The scratch area was examined using Image J software to calculate the average cell scratch mobility. The average scratch mobility (%) was calculated as follows:( scratch area at 0 h - scratch area at 24 h or 48 h)/0 h scratch area × 100%.

3.8 Flow cytometry

After 24 h of transfection, OGCs were collected and washed twice with PBS, and 1 mL of pre-cooled 75% ethanol was added slowly to the cell precipitate to resuspend the cells, which were fixed overnight at 4 ℃. On day 2, the overnight fixed cells were centrifuged at 1,500 rpm for 5 min, washed once with PBS, followed by resuspension in 100.0 μL of PBS, and 2.0 μL of 10 mg/mL RNase A (MCE, Shanghai, China), and incubated in a water bath at 37 °C for 30 min. Propidium iodide staining solution (100 μg/mL; MCE, Shanghai, China) was added to the cells and stained for 10 min under low light conditions. The cells were then detected by flow cytometry (CytoFLEX S, Beckman Coulter, China) at the excitation and emission wavelengths of 488 nm and 585 nm, respectively, and then the cell cycle was analyzed by Modfit software to determine the cell cycle distribution.

3.9 Western blotting

MiRNA-98 mimics and NC mimics were transfected in OGCs using siRNA-mate. After 24 h of transfection, cells were lysed using a pre-cooled RIPA lysis buffer (ApplyGen, Beijing, China) mixed containing 0.1 mg/mL phenylmethylsulfonyl fluoride (Thermo Fisher Scientific, Shanghai, China). The mixture was centrifuged at 4°C, total cellular proteins was collected, and the concentration was determined by the BCA kit (Solarbio, Beijing, China). Total protein extracts were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and blotted on polyvinylidene difluoride (Millipore, Billerica, USA) membranes. The membranes were blocked with 5% skimmed milk at 4°C for 2 h. At the end of the incubation period, the membranes were incubated overnight with specific primary antibodies at 4°C, followed by a secondary antibody for 1.5 h. The membranes were treated with ELC developer (solution A: solution B = 1:1) (Beyotime, Shanghai, China), and chemiluminescence was detected employing the Universal Hood II (BIO-RAD, USA) system. The protein grayscale was analyzed by Image J software. Primary antibodies used in this assay were as follows: BAX (1:1000, Proteintech,

Wuhan, China), proliferating cell-nuclear antigen (PCNA, 1:1000; Proteintech, Wuhan, China), B-cell lymphoma 2 (BCL2, 1:2000; Abcam, USA), caspase 3 (1:1000, Cell Signaling Technology, Carlsbad, CA, USA), caspase 9 (1:2000, Abcam, USA), 3β-hydroxysteroid dehydrogenase (3β-HSD, 1:2000; Proteintech, Wuhan, China),cytochrome P450 17A1 (CYP17A1, 1:2000; Proteintech, Wuhan, China),COL1A1(1:2000;Proteintech, Wuhan, China),glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000; Proteintech, Wuhan, China).

3.10 Enzyme-linked immunosorbent assay (ELISA)

After 24 h of cell transfection, cell supernatants were collected, and E2 levels were detected using an ELISA kit (Kexing, Shanghai, China). The absorbance of different treatment groups was determined at 450 nm using an enzyme labeling instrument (BioTek) following the kit instructions. The concentrations of corresponding samples were estimated by linear regression of the standard curve.

3.11 Data analysis

All the experimental results in this study had been subjected to at least three repetitions. The results are expressed as mean ± standard deviation. The results of the cell scratching experiments were examined using Image J software for gray value analysis of the intended bands. Data were processed using SPSS 19.0, and multiple group comparisons were analyzed by analysis of variance. Comparisons between the two groups were performed using an independent samples t-test before Shapiro-Wilk test and Levene's test,and plotted using GraphPad Prism 9.5. In the analysis of multiple comparisons, different letters in the upper case indicate significant differences ($P<0.05$), while the same letters also indicate differences without statistical significance ($P<0.05$). The results of multiple comparisons are corrected by Bonferroni correction.



## 4. Results and analysis

4.1 Potential target prediction of miRNA-98 with COL1A1/3'-UTR

Using TargetScan8.0 BioOnline software, five miRNAs with the highest scores for regulating the expression of the *COL1A1* gene were obtained (Figure 1-A and B). These were miR-29d-3p, miR-218, miR-98, miR-133ab, and miR-3. Analysis of the expression patterns of the five miRNAs in the ovaries of single and multiple lambs of Guizhou black goats revealed that both the single and multiple lamb groups had high expression of miR-29-3p, but there was no significant difference. In contrast, a significantly lower expression of miR-31 was noted in the single lamb group than the multiple lamb

group ($P < 0.05$), and the same trend was observed for miR-218 and miR-133 ($P < 0.01$), whereas the expression of miR- 98 in the single-lamb group was highly significant higher compared to that in the multi-lamb group ($P < 0.01$) (Figure 1-C). The expression of miR-98 was enhanced in the ovaries of the single-lamb group of ewes compared with those in the multi-lamb group. Moreover, the expression of *COL1A1* in the ovaries of the multi-lamb group was significantly higher than that of the single-lamb group. It was, therefore, hypothesized that miR-98 might be the optimal miRNA for the targeted regulation of the *COL1A1* gene expression.

4.2 miR-98 targets and inhibits *COL1A1* expression

Using the bioinformatics software Targetscan, a reciprocal binding site for miR-98 and COL1A1 3'-UTR was predicted at 789~793 (Figure 2-A). The 3'-UTR 70 bp fragment of the *COL1A1* gene harboring the region of the interaction was selected to construct the vector. The constructed pGL-WT-COL1A1/3'-UTR wild-type vector and pGL-MT-COL1A1/3'-UTR mutant vector were confirmed by double digestion, which confirmed the expected length of the *COL1A1* gene target fragment (around 80 bp) and the pmirGLO vector fragment (7,350 bp) of the wild-type and mutant vectors (Figure 2-C). The sequencing results further established that the sequence of the wild-type vector was consistent with that of the reference sequence. In the mutant vector, MT-COL1A1, the sequence " UACCU" was changed to "ATGGA," and the results were as expected (Figure 2-D). The subsequent experiments were performed using these constructs. Dual luciferase assay showed significantly lower luciferase activity of HEK-293t cells transfected with WT-COL1A1/3'-UTR+miR-98-mimics group than that of WT-COL1A1/3'-UTR+NC-mimics-transfected and WT-COL1A1/3'-UTR+miR-98-inhibit-transfected cells(*P<0.01)*. However, there were no significant differences between the other groups, except for WT-COL1A1/3'-UTR+miR-98-mimics group(Figure 2-E).The RT-qPCR results further showed that miR-98 overexpression down-regulated the mRNA expression of *COL1A1* at high significance ($P < 0.01$) (Figure 2-F). The overexpression of miR-98 significantly reduced BCL2 expression ($P < 0.01$) (Figure 2-G)

4.3 Overexpression of miR-98 inhibits proliferation and migration of ovarian granulosa cells

The miR-98 mimics and NC mimics were transfected into ovine OGCs, and the efficiency of transfection was verified through qRT-PCR. As shown in Figure 3, after 24 h of transfection, miR-98 expression in the transfected group was extremely significantly higher than that in the NC group *(P < 0.01)* (Figure 3-A), indicating the expected transfection efficiency, and the CCK8 and cell scratch experiments could be performed on the OGCs. The CCK-8 results showed that miR-98 overexpression

highly significantly inhibited OGCs proliferation when transfected with miR-98 for 72 h *(P < 0.01)* (Figure 3-B). The cell scratch assay revealed that the area of migration of OGCs in the miR-98-transfected experimental group was significantly lower than that in the NC group *(P < 0.05)* (Figure 3-C). It indicated that miR-98 overexpression significantly inhibited the proliferation and migration ability of OGCs.

4.4 miR-98 Overexpression inhibits the normal functioning of the cell cycle and expression of related genes in ovarian granulosa cells

The effect of miR-98 on OGCs cell cycle was examined by flow cytometry. After miR-98 overexpression, the proportion of OGCs in the G1 and G2 phases increased significantly ($P < 0.05$), while the percentage of cells in the S phase decreased significantly ($P < 0.01$) (Figure 4-A, B). Further examination of the expression of cell-cycle-related genes *CDK1*, *CDK2*, and *CCNE* by RT-qRCR revealed that miR-98 overexpression highly significantly downregulated *CDK1* expression ($P < 0.01$), and also significantly down-regulated the expression of *CDK2* and *CCNE* mRNAs ($P < 0.05$) (Figure 4-C).

4.5 Overexpression of miR-98 promotes apoptosis in ovarian granulosa cells

The levels of inhibitory apoptotic protein BCL2 and pro-apoptotic proteins BAX, caspase3, and caspase9 were determined by western blotting. The overexpression of miR-98 highly significantly suppressed BCL2 expression ($P < 0.01$) (Figure 5), but significantly up-regulated the expression of BAX and caspase9 ($P < 0.01$), and the same trend was noted for caspase3 ($P < 0.05$).

4.6 Overexpression of miR-98 inhibits the secretion of ovarian granulosa cells $E_2$ and secretion levels of genes related to hormone synthesis

The effect of miR-98 overexpression on $E_2$ levels in the culture medium of OGCs was detected by ELISA. The overexpression of miR-98 highly significantly inhibited $E_2$ secretion (Figure 6-A). Further, qRT-PCR assay showed that the relative mRNA expression of genes related steroid hormone synthesis, including *CYP11A1*, *CYP19A1*, and *FSHβ* was down-regulated after miR-98 overexpression in OGCs at high significance ($P < 0.01$) (Figure 6-B). The results of western blotting showed that miR-98 overexpression extremely significantly suppressed the expression of CYP17A1 and 3 β -HSD proteins ($P < 0.01$) (Figure 6-C).

**5. Discussion**

5.1 Effect of the *COL1A1* gene on reproductive performance of experimental animals

Follicular development requires interactions among signaling from oocytes, membrane cells, granulosa cells, and enveloping stromal components, including blood vessels and the immune system (28). The proliferation and differentiation of OGCs lead to the maturation and ovulation of follicles, whereas OGC apoptosis and degeneration lead to follicular atresia (5, 6), hence, the apoptosis of OGCs is considered to be a marker of follicular atresia (7). Collagen is an important component of the (ECM) and participates in the maintenance of tissue and organ structure, as well as the proliferation and migration of cells. The enhanced expression of *COL1A1*, an important gene for type I collagen, contributes to type I collagen deposition in luteinizing follicular cells and promotes angiogenesis in the ovaries (17). Furthermore, its expression is linked to morphological changes in the structural organization [24] and the development of bovine fetal ovary (29). The study on the main genes for follicular maturation, ovulation, and regression in pigeons revealed that *COL1A1* is an important candidate gene involved in promoting preovulatory follicular maturation and ovulation by participating in the ECM receptor interaction pathway (23). Our previous studies on goats found a positive correlation of the *COL1A1* gene with lambing traits, and goat ovarian OGCs that interfere with the expression of the *COL1A1* gene severely affected proliferation and apoptosis in OGCs, indirectly affecting follicular development and maturation in goats. The above findings suggest that different expression levels of the *COL1A1* gene is closely associated with follicular development and oocyte maturation in mammals.

5.2 Effect of miR-98 on ovarian granulosa cell proliferation and apoptosis

miR-98 is a member of the Let-7 family, widely involved in the physiological processes of cell growth, apoptosis, migration, and invasion in various cancer cells. Hui et al. found that miR-98-5p promotes apoptosis and inhibits migration of the cells by suppressing of signal transducer and activator of transcription 3 protein expression (27) . Zheng et al. found that miR-98 inhibits cell proliferation, migration, and invasion by suppressing *CLDN1* expression in human colon cancer and promotes apoptosis. miR-98 also targets high-mobility group AT-hook 2 (HMGA2) protein to inhibit breast cancer cell proliferation, invasion, and migration and promote apoptosis (15). Likewise, Zheng et al. found that miR-98 inhibits cell proliferation, migration, and invasion, and promotes apoptosis in human colon cancer by downregulating *CLDN1* (28), showing that miR-98 plays a role similar to that of oncogenes in various types of cancer cells. In addition, miR-98 also plays a role in regulating the development of diseases of the ovary and influencing follicular development. Zhu et al. found that miR-98 up-regulates the inhibition of proliferation, activity, and migration of human trophoblast cell HTR-8/SVneo, which

may because miR-98 targets *GDF6* and *FAPP2* (31)[]. Xu et al. studied the effect of silica nanoparticles (NPs) on germ cells, and found that different expression levels of miR-98 play a key role in silica NP-induced loss of mitochondrial membrane potential and apoptosis in germ cells (32). In addition, in their rat embryo implantation study, Xia et al. found that a decrease in the expression of miR-98 promoted embryonic stem cell proliferation and inhibited apoptosis, whereas its enhanced expression had the opposite effect, a process related to miR-98 targeting BCL-xl (30). In the current study, we found that miR-98, as one of the candidate miRNAs regulating the *COL1A1* gene expression, was significantly down-regulated in the ovaries of the multiple lambing group ewes, contrary to *COL1A1* gene expression in the above-mentioned tissues. Therefore, we hypothesized that miR-98 indirectly affects the reproductive performance of goats by targeting and regulating the expression of the COL1A1 gene.To confirm this hypothesis, we first used a dual luciferase reporter gene assay and confirmed that miR-98 can target the "CUACCUC" site in the 3'-UTR region of the *COL1A1* gene and regulate the *COL1A1* gene expression. Further, the overexpression of miR-98 inhibited OGC proliferation and migration, significantly down-regulating the level of PCNA protein and the Bcl-2/Bax ratio, and up-regulated the level of caspase-3 and caspase-9 proteins, suggesting that miR-98 can target and regulate *COL1A1* gene expression and further inhibit the expression of COL1A1 protein.What's more,miR-98 play an inhibitory role in proliferation and migration of OGCs in goats. This is consistent with the findings of Zhu, Xu, and Xia et al. in human trophoblasts, germ cells, and embryonic stem cells.

5.3 Effects of proliferation and apoptosis of ovarian granulosa cells on steroid hormone synthesis and secretion

OGCs are the main source of steroid hormone synthesis and secretion. Hence, their proliferation and apoptosis are bound to significantly impact steroid hormone synthesis and secretion. E2 produced in OGCs can support the survival and proliferation of these cells, promote follicular maturation, and induce follicular atresia if insufficiently secreted(33). In contrast, 3 β -HSD, *CYP11A1*, *CYP17A1*, *CYP19A1*, and *FSH β* are crucial for the synthesis of E2(34, 35). Recent studies have found that $E_2$ is involved in regulating follicular development through binding to ESRs (ESR1 and ESR2), and that hypoxia treatment to buffalo OGCs activates the cAMP/PKA signaling pathway and promotes the expression of genes related to $E_2$ synthesis (*CYP11A1*, *CYP19A1*, and *3 β-HSD*) and levels of E2 in OGCs(36). Li et al. found that miR-146b inhibits estradiol synthesis in OGCs by targeting the *CYP19A1* gene, which promotes apoptosis and follicular atresia(37). Furthermore, Zhang et al. found that miR-31 and miR-143 inhibit

the synthesis and secretion of progesterone (P4) and estrogen (E2) in OGCs and apoptosis in OGCs by targeting follicle-stimulating hormone receptor(19). To investigate the effect of miR-98-targeted regulation of the *COL1A1* gene, we detected the expression of E2 and steroid synthesis-related genes, *3β-HSD*, *CYP17A1*, *CYP11A1*, and *CYP19A1*, and the levels of proteins by ELISA, qRT-PCR, and western blotting. Overexpression of miR-98 in OGCs led to a decrease in E2 secretion in the culture medium and down-regulation suppression of the expression of 3β-HSD, CYP11A1, CYP17A1, CYP19A1, and FSH β in OGCs. The overexpression of miR-98 can, thus inhibit $E_2$ secretion by down-regulating the expression of the above-mentioned steroid synthesis-related genes, in turn affecting the proliferation and migration of OGCs and promoting their apoptosis.

**6. Conclusion**

This study shows that miR-98 can specifically bind to the 3'-UTR of *COL1A1* to inhibit the expression of the *COL1A1* gene in Guizhou black goats *(P<0.01)*. In addition, overexpression of miR-98 could significantly suppress the proliferation, migration, and estradiol secretion ability of OGCs *(P<0.05)* and inhibit the expression of genes associated with proliferation markers, steroid secretion, and pro-apoptosis.

## 7. Acknowledgments

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## 8. Disclosure

All authors have no competing interests to declare.

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490 **10.Tables and Figures**

491 **Table 1 Sequence number of mRNA and miRNA**

| Gene name | Sequence(5'-3') | Number |
|---|---|---|
| bta-miR-29d-3p | UAGCACCAUUUGAAAUCGAUUUA | MIMAT0009275 |
| bta-miR-218 | UUGUGCUUGAUCUAACCAUGUG | MIMAT0003798 |
| bta-miR-98 | UGAGGUAGUAAGUUGUAUUGUUU | MIMAT0003809 |
| bta- miR-133a | UUUGGUCCCCUUCAACCAGCUG | MIMAT0009225 |
| bta-miR-31 | AGGCAAGAUGCUGGCAUAGCU | MI0004762 |
| Col1a1 | | XM_018064893.1 |
| GAPDH | | XM_005680968.3 |
| U6 | | NR_004394.1 |

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495 **Table 2 Specific neck ring primers for miRNA reverse transcription**

| Gene name | Sequence (5' to 3') |
|---|---|
| Stem loop sequence | GGTCGTATGCAAAGCAGGGTCCGAGGTATCCATCGCAC GCATCGCACTGCATACGACC |
| RT-miR-29d-3p | GGTCGTATGCAAAGCAGGGTCCGAGGTATCCATCGCAC GCATCGCACTGCATACGACCTAATCGAT |
| RT-miR-218 | GGTCGTATGCAAAGCAGGGTCCGAGGTATCCATCGCAC GCATCGCACTGCATACGACCCACATGGT |
| RT-miR-98 | GGTCGTATGCAAAGCAGGGTCCGAGGTATCCATCGCAC GCATCGCACTGCATACGACCAACAATAC |
| RT-miR-133a | GGTCGTATGCAAAGCAGGGTCCGAGGTATCCATCGCAC GCATCGCACTGCATACGACCCAGCTGGT |
| RT-miR-31 | GGTCGTATGCAAAGCAGGGTCCGAGGTATCCATCGCAC GCATCGCACTGCATACGACC **AGCTATGC** |
| R-U6 | AACGCTTCACGAATTTGCGT |

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499 **Table 3 qRT-PCR primers used in this study**

| Gene name | Sequence (5' to 3') | Amplified length (bp) |
|---|---|---|
| miR-29d-3p /Q-R | TAGCACCATTTGAAATCGATTAG | 71 |
| miR-218/Q-R | TTGTGCTTGATCTAACCATGTG | 71 |
| miR-98/Q-R | TGAGGTAGTAAGTTGTATTG | 71 |
| miR-133a/Q-R | TTTGGTCCCCTTCAACCAGC | 71 |
| miR-31/Q-R | AGGCAAGATGCTGGCATAG | 71 |
| miR-Universal/Q-F | CAAAGCAGGGTCCGAGGTATC | 71 |
| U6-F | CTCGTTCGGGCAGCACA | 83 |
| U6-R | AACGCTTCACGAATTTGCGT | |
| Col1a1-F | AAGAAGAAGACATCCCCACCAG | 102 |
| Col1a1-R | CAGATCACGTCATCGCACA | |
| CCNE1-F | AAGTGCTCCTGCCTCAGTATCCTC | 122 |
| CCNE1-R | ATACAAGGCGGAAGCAGCAAGTA | |
| CDK1-F | AGGGTAGACACAAAACTACAGG | 91 |
| CDK1-R | TGCAGTACTAGGAACCCCTT | |
| CDK2-F | ATCCGCCTGGACACTGAG | 87 |
| CDK2-R | GTAGGAGGACCCGATGAGA | |
| CYP11A1-F | CGGACAAGTTTGACCCAACCAG | 144 |
| CYP11A1-R | GCCGGAAGACAAGGAAGATGG | |
| CYP19A1-F | TCCAGTGAGCAGCAGGATTG | 89 |
| CTP19A1-R | TCCGTAAGCCGAGAATG | |
| FSHβ-F | AGATGTCTGTGTGTACATGCG | 122 |
| FSHβ-R | GGTTGGGCTCTCTTCTCTCTTGAGG | |
| β -actin-F | GGTGCCCATCTACGAGG | 154 |
| β -actin-R | CTTGATGTCACGGACGATT | |

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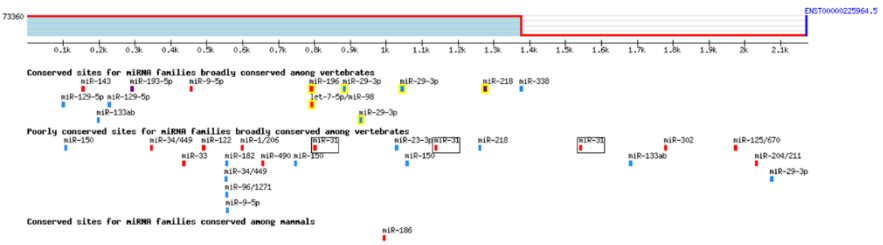
A




B

| miRNA family | Conserved sites | | | | Poorly conserved sites | | | | 6mer sites | Cumulative weighted context++ score | Total context++ score | Aggregate $P_{CT}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total | 8mer | 7mer-m8 | 7mer-A1 | Total | 8mer | 7mer-m8 | 7mer-A1 | | | | |
| miR-29-3p | 3 | 0 | 0 | 3 | 1 | 0 | 0 | 1 | 0 | -1.67 | -2.08 | > 0.99 |
| miR-218 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | -0.41 | -0.42 | 0.86 |
| let-7-5p/miR-98 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | -0.29 | -0.29 | 0.89 |
| miR-133ab | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | -0.27 | -0.28 | 0.67 |
| miR-31 | 0 | 0 | 0 | 0 | 3 | 0 | 3 | 0 | 1 | -0.20 | -0.27 | 0.23 |

C

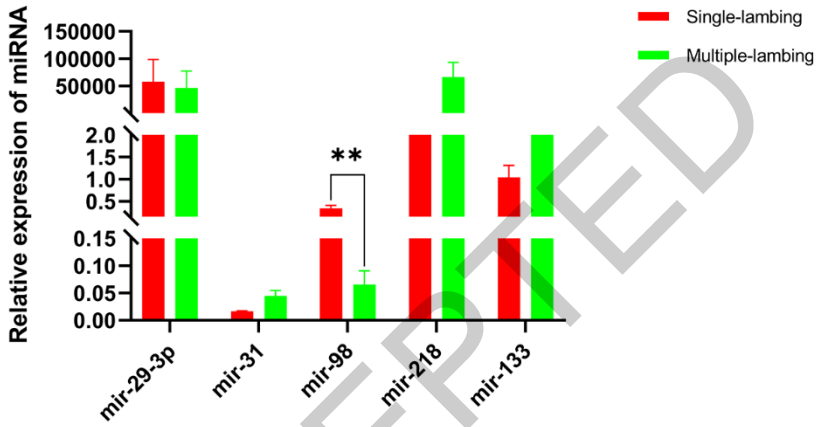



**Fig.1 Screening the best miRNAs that target and regulate *COL1A1*.**(A) Screening of miRNAs regulating *COL1A1* gene using TargetScan8.0(B) Score map of miRNAs regulating *COL1A1* gene expression (C) Using RT-qPCR to detect the expression of candidate miRNA in the ovaries of single-lamb black goats and multi-lamb black goats.

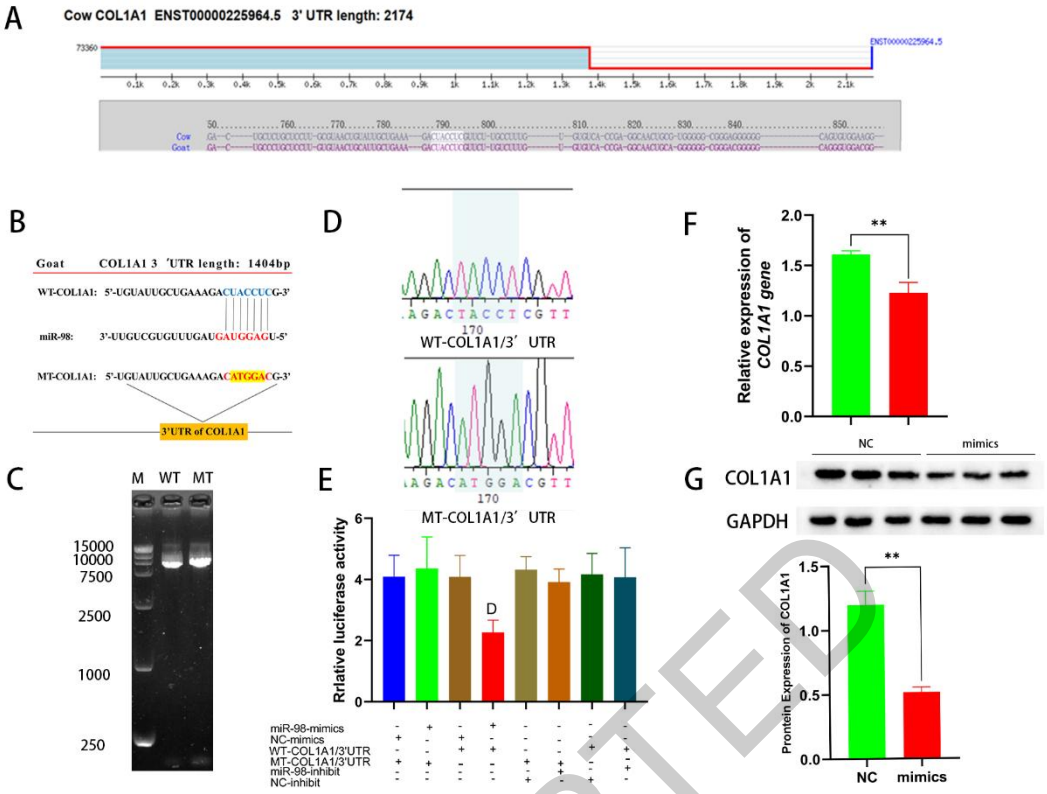



**Fig.2 Direct regulation of COL1A1by miR-98 in OGCs.** (A) Use the website to find a high degree of homology between cattle and goat (B) The wildtype and mutant type sequences of the COL1A1 gene in the 3' non-coding region (C) Identification result of enzyme digestion (D) Squencing results of wild type and mutant type vecto (E) Dual-luciferase reporter assays in OGCs using vectors encoding a putative miR-98 target site in the COL1A13′-UTR (positions 789~793). Data were normalized to the Renilla/firefly expression ratios. Same capital letters indicate no significant differences *(P<0.05)*; Different capital letters indicate significant differences *(P<0.05)*. (F)RT-qPCR detection of *COL1A1* gene expression in OGCs after miR-98 overexpression. (F) Protein expression of *COL1A1* in OGCs after miR-98 overexpression. “＊＊” The difference is extremely significant *(P<0.01)*.

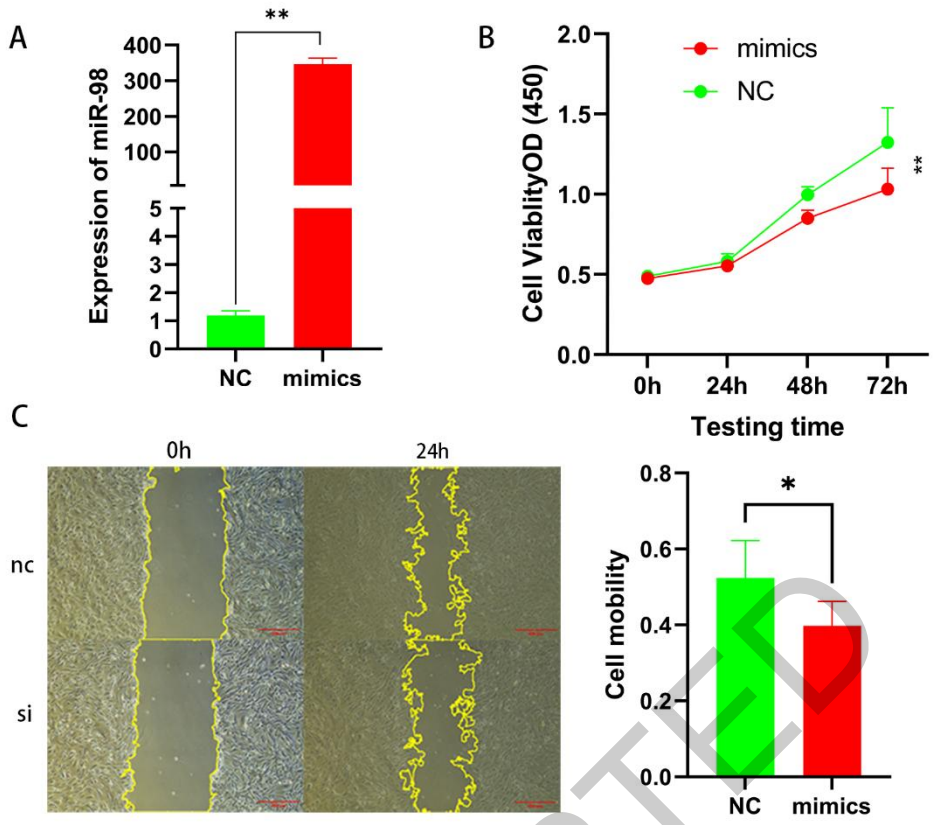



**Fig.3 The overexpression of miR-98 inhibits the proliferation and migration of OGCs.** (A) The transfection efficiency of miR-98 mimic inhibitor in OGCs lines were assessed by qRT-PCR at 24 hours post transfection (hpt). (B) The effects of miR-98 overexpression on the proliferation of OGCs. The CCK-8 assay showed that the overexpression of miR-98 significantly inhibited cell proliferation compared with miR-98. The CCK-8 assay showed that the overexpression of miR-98 significantly inhibited cell proliferation compared with the NC mimics at 72 hpt ($P<0.01$). (C) Representative images of migration assays using OGCs. magnification, 100×. Quantification of relative migration of OGCs transfected with miR-98 mimics and NC mimics. "＊"The difference is significant *($P < 0.05$)*. "**"The difference is extremely significant *($P<0.01$)*.

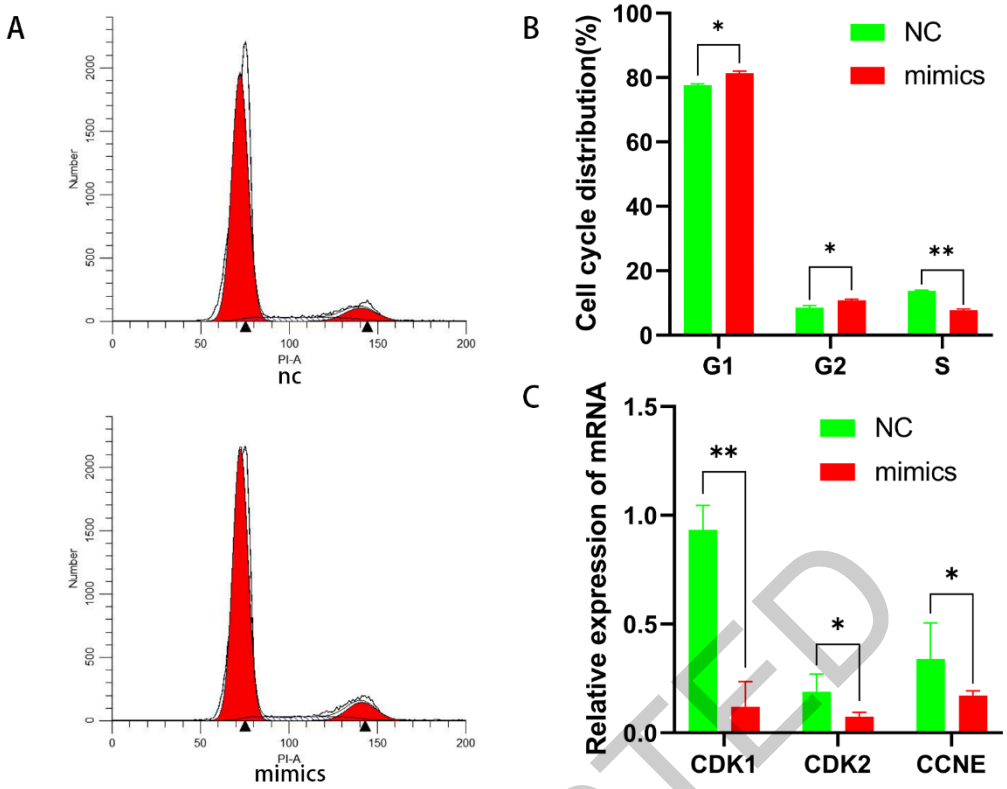



**Fig.4 The impact of miR-98 overexpression on the OGCs cycle.** (A) Using flow cytometry to detect the cycle distribution of OGCs after overexpressing miR-98. 98. (B) Cell cycle distribution of OGCs after overexpressing miR-98.(C) RT-pCR was used to detect the expression of cell cycle-related genes CDK1, CDK2 and CDNE in OGCs cells after miRNA overexpression. "＊"The difference is significant *(P < 0.05)*. "＊＊" The difference is extremely significant *(P<0.01)*.

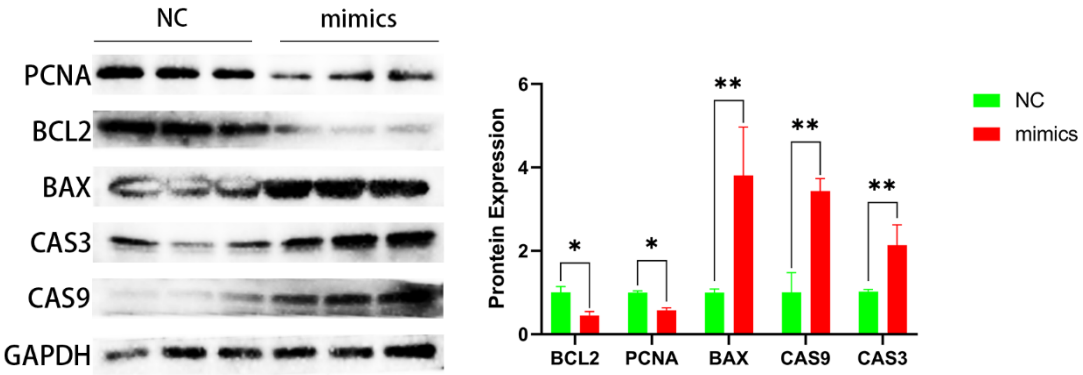



Fig.5 Western blot analysis was performed to evaluate the expression of PCNA, BCL2, BAX, and CAS3 in OGCs treated with miR-98 and NC. "*"The difference is significant *(P<0.05)*. "**"The difference is extremely significant *(P<0.01)*.

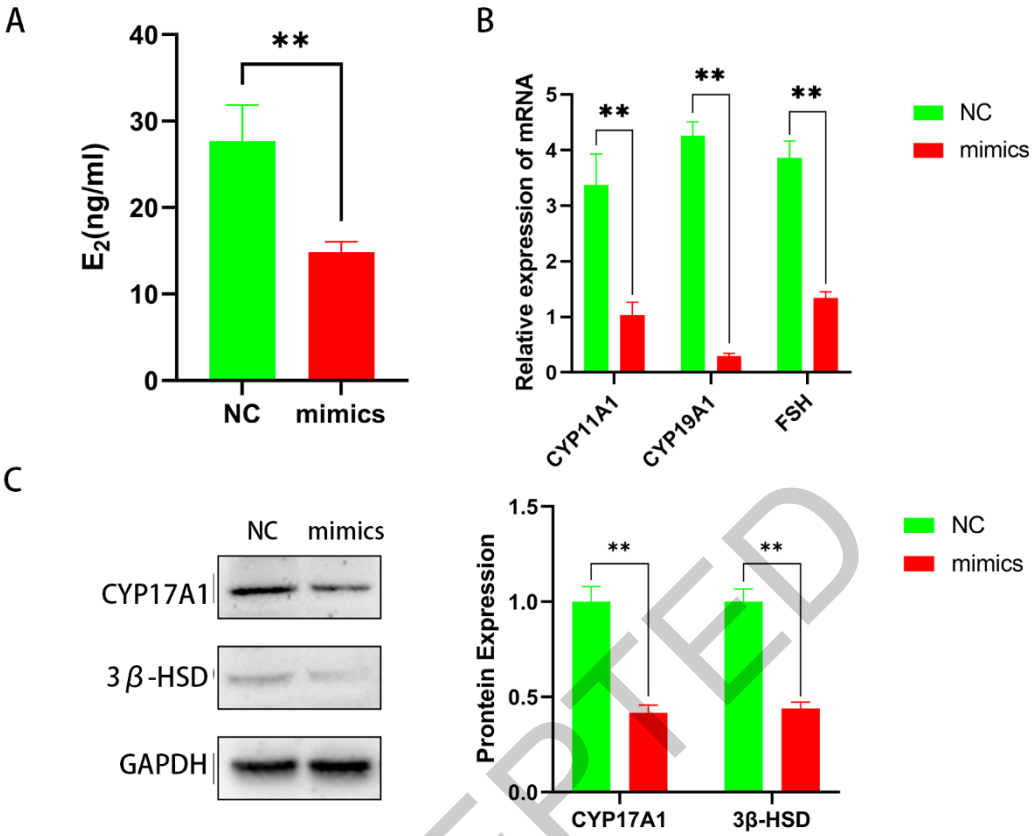
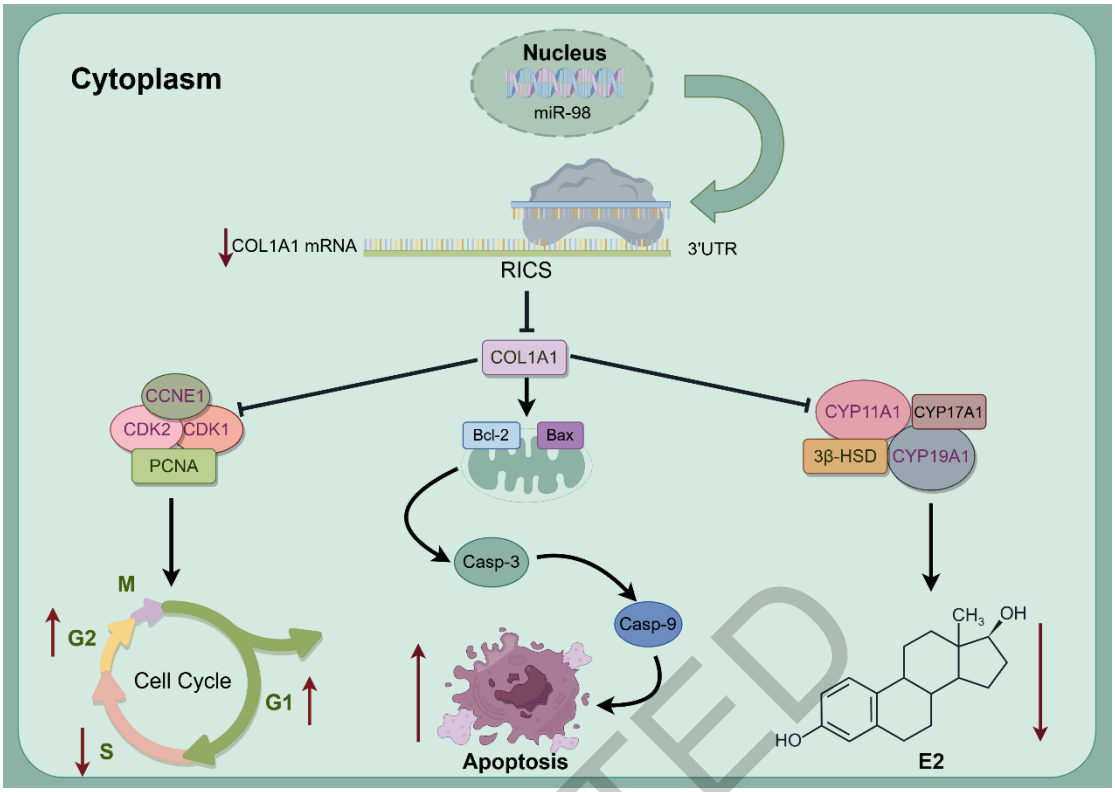



**Fig.6 Overexpression of miR-98 inhibits E2 secretion by OGCs.**(A) ELISA kit detects E2 levels in culture medium after transfection with miR-98 or NC(B) RT-pCR was used to detect the expression of steroid secretion related genes CYP11A1, CYP19A1 and FSHβ in OGCs cells after miRNA overexpression.(C) Western blot analysis was performed to evaluate the expression of CYP17A1and 3 β -HSD in OGCs treated with miR-98 and NC. "**" The difference is extremely significant *(P<0.01)*.

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553 Fig.7 The mechanism of miR-98 regulating estradiol synthesis,proliferation and apoptosis in goat

554 ovarian granulosa cells.

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