COL1A1 may be associated with lambing traits in goats by regulating the biological function of ovarian granulosa cells via the PI3K/AKT/mTOR signalling pathway

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Abstract

Collagen type I alpha 1 chain (COL1A1), an important component of type I collagen protein, plays a role in regulating follicle development and ovarian cell proliferation, which is closely related to animal reproductive traits. The aim of this study was to explore the relationship between the expression of COL1A1 and lambing traits of Guizhou black goats (Capra hircus), and regulatory mechanisms in follicle development. Results showed that the protein expression level of COL1A1 in ovarian tissues of the polytocous group were higher than those of the monotocous group, and COL1A1 was expressed in all follicles and ovarian cells. Upregulation of COL1A1 significantly promoted cell proliferation, migration and the development of cells from G1 to G2/M phase, reduced intracellular ROS levels. Additionally, COL1A1 overexpression resulted in significantly increased expression of proliferating cell nuclear antigen (PCNA) and the ratios of p-PI3K (phosphorylation-phosphatidylinositol 3-kinase)/PI3K, p-AKT (phosphorylation-protein kinase B)/AKT and p-mTOR (phosphorylation-mammalian target of rapamycin)/mTOR, and decreased expression of BCL2-Associated X (BAX), caspase-3 and caspase-9. The reverse result, however, was seen when COL1A1 expression was downregulated in granulosa cells. Taken together, COL1A1 was widely and highly expressed in ovarian tissues of polytocous goats, and it may participate in regulations of lambing traits by regulating the proliferation and migration of granulosa cells by activating the PI3K/AKT/mTOR signalling pathway and reducing ROS production in granulosa cells to facilitate the development and maturation of follicle.

1 Introduction

In mammals, female reproductive performances depend on the ovarian healthy growth. Genetic variations in the ovaries have a significant impact on follicle development, ovulation, oestrogen release, and litter size. The proliferation of granulosa cells, the increase of cell layers, and the expand in follicle volume are the principal manifestations of changes in follicle morphology throughout follicle development. Granule cells secrete hormones, cytokines, and proteins that control the growth and development of follicles while also supplying oocytes with nourishment. A condition known as follicular atresia causes the majority of follicles to cease growing and deteriorate. Follicle atresia is directly caused by granulosa cell apoptosis. Additionally, ovarian-derived disorders such polycystic ovary syndrome, premature ovarian failure, and impaired maternal fertility can be brought on by the premature death of granulosa cells. It has been proved that the proliferation, apoptosis and cycle of granulosa cells are important factors affecting the number of lambs produced by goats. Hence, it is crucial and significative to understand underlying mechanism of regulation ovarian granulosa cells on prolificacy in goats.

Collagen is an important component in the formation of extracellular matrix (ECM), which involves in tissue remodeling and morphogenesis and follicle development, including type I, II, and III collagen. Increased evidences revealed that collagen type I alpha 1 chain (COL1A1), a vital component of type I collagen, has been shown to play a function in cell proliferation, invasion, metastasis, and angiogenesis.
In clinical researches, COL1A1 has been reported linked to the tumorigenesis of several cancers, such as laryngeal cancer\textsuperscript{12}, colon cancer\textsuperscript{13} and ovarian cancer\textsuperscript{14}. Moreover, COL1A1 plays an important role in maintaining normal ovarian function, Follicle-stimulating hormone (FSH) and menopausal gonadotropin have been discovered to have specific regulatory effects on COL1A1, which is critical for cell assembly and formation\textsuperscript{15,16}, and exhibits differential expression in follicular atresia\textsuperscript{17}. Research on bovine cumulus cells has revealed that COL1A1 contributes to preserving follicle development and ovarian expansion\textsuperscript{18}. These data implied that COL1A1 may affect the reproductive performances by regulating follicle development.

The protein kinase B (PKB/Akt) activated by phosphatidylinositol-3-kinase (PI3K) acts on mammalian sirolimus target protein (mTOR) and downstream effective effector, and has crucial for regulating angiogenesis, proliferation, apoptosis, growth movement, survival, and metabolism\textsuperscript{19}. Data from ovarian cancer showed that the PI3K/AKT/mTOR pathway was active in roughly 70\% of cases\textsuperscript{20} and is critical for ensuring the survival of healthy ovarian cells\textsuperscript{21}. Additionally, upregulation of PI3K/AKT/mTOR pathway can promote follicle development and obtain an increase in follicle proliferation since follicle atresia can be avoided by using the PI3K inhibitor LY294002\textsuperscript{22}.

So far it is still unknown whether the effect of the COL1A1 gene on goat ovarian development is also achieved by regulating PI3K/AKT/mTOR. Therefore, the aim of this study is firstly to investigate the correlation between the expression of COL1A1 and lambing traits of Giuzhou black Goats. Furthermore, ovarian granulosa cells were used to elucidate the effect of PI3K/Akt/mTOR signalling pathway on the regulation of COL1A1 on lambing traits in goats.

2 Results

2.1 Protein expression and localization of COL1A1 in ovarian tissues of monotocous and polytocous groups

The results of immunohistochemistry showed that COL1A1 protein was widely expressed in ovarian tissues of polytocous goats, was distributed in the theca cells and granulosa cells of primordial follicles, tertiary follicles and mature follicles and was widely expressed in connective tissues(Fig. 1a). The positive area ratio (Fig. 1f) and H-score (Fig. 1d) analysis results showed that the positive area ratio, the number of positive cells and the fluorescence intensity of COL1A1 protein in the ovarian tissue of the polytocous goat group were higher than those of the monotocous goat group \((P < 0.01)\). Western blotting showed the same results as immunohistochemistry (Fig. 1b c). Furthermore, morphological observation revealed that ovarian tissues of the polytocous group had larger volumes and more mature follicles than those of the monotocous group (Fig. 1e).

2.2 Cell identification and localization of COL1A1 in granulosa cells
Since follicle-stimulating hormone receptor (FSHR) was only specifically expressed in granulosa cells, the immunofluorescence can be used to confirm the cell type in ovarian follicles. After the cells were stained with FSHR, 90% of the cells gave off red light (Fig. 2), indicating that the isolated and cultured cells were ovarian granulosa cells with high purity. Immunofluorescence was used to determine the localization of COL1A1 protein in granulosa cells. The results showed that COL1A1 protein was located in the cytoplasm and nucleus of granulosa cells (Fig. 2).

2.3 COL1A1 promotes cell migration and has reduces ROS

To detect the expression efficiency of COL1A1 protein, granulosa cells were transfected with sh-COL1A1, sh-NC, pcDNA3.1(+) -COL1A1 and pcDNA3.1(+) vectors for 48 h. Western blotting was performed to detect the expression of COL1A1 protein in different transfection groups (Fig. 3a). The results showed that the expression of COL1A1 protein in the sh-COL1A1 group was significantly lower than that in the sh-NC group (P < 0.05), and the expression of COL1A1 protein in the pcDNA3.1(+) -COL1A1 group was significantly higher than that in the pcDNA3.1(+) empty vector group (P < 0.05) (Fig. 3b). These results indicated that the COL1A1 gene interference and overexpression vectors were successfully constructed and correctly expressed. Then, different expression vectors of COL1A1 were transfected again to detect their effects on ROS (Fig. 3c) and cell migration (Fig. 3d) of granulosa cells. The results showed that compared with the sh-NC group, the migration distance of granulosa cells in the sh-COL1A1 group was reduced (P < 0.05) (Fig. 3e), and the intracellular ROS level was increased (Fig. 3c). Compared with the pcDNA3.1(+) group, pcDNA3.1(+) -COL1A1 group was the migration distance of granulosa cells increased (Fig. 3e) and the intracellular ROS level decreased (P < 0.05) (Fig. 3c). These results suggest that upregulation of COL1A1 can significantly reduce intracellular ROS levels and promote cell migration, and vice versa.

2.4 COL1A1 promotes granulosa cell proliferation and cell cycle progression

To detect the effect of different expression levels of the COL1A1 gene on cell proliferation, CCK8 and flow cytometry were used to detect the effects of transfection of sh-COL1A1, sh-NC, pcDNA3.1(+) -COL1A1 and pcDNA3.1(+) on the proliferation and cycle of granulosa cells. Western blotting was used to detect the effects of different expression levels of the COL1A1 gene on the expression levels of the pro-proliferative protein PCNA and the pro-apoptosis-related proteins BAX, caspase-3 and caspase-9 (Fig. 4a). The results showed that the proliferation ability of granulosa cells in the transfected pcDNA3.1(+) - COL1A1 group was significantly higher than that in the pcDNA3.1(+) group (P < 0.01) (Fig. 4f) and promoting the development of granulosa cells from G1 to G2/M phase (Fig. 4g). At the same time, it promoted the expression of PCNA (Fig. 4b) protein and inhibited the expression of BAX (Fig. 4c), caspase-3 (Fig. 4d), and caspase-9 protein (Fig. 4e). In contrast, the proliferation ability of granulosa cells in the sh-COL1A1 group was significantly lower (P < 0.01) (Fig. 4f), and kept granulosa cells in G1 phase (Fig. 4g) than that in the sh-NC group, the expression of PCNA (Fig. 4b) protein was inhibited, and the expression of BAX
(Fig. 4c), caspase-3 (Fig. 4d) and caspase-9 (Fig. 4e) protein was promoted. The above results suggest that the expression level of the COL1A1 gene is positively correlated with the proliferation of granulosa cells.

2.5 COL1A1 activates the PI3K/AKT/mTOR pathway of granulosa cells

To study the role of the COL1A1 gene in the cell proliferation pathway PI3K/AKT/mTOR, different expression vectors of the COL1A1 gene were transfected into granulosa cells, and total cell proteins were extracted for Western blot assays (Fig. 5a). The results showed that the ratios of p-PI3K/PI3K (Fig. 5b), p-AKT/AKT (Fig. 5c) and p-mTOR/mTOR (Fig. 5d) in the pcDNA3.1(+)-COL1A1 transfected group were significantly higher than those in the pcDNA3.1(+) empty vector group (P < 0.05). However, the ratios of p-PI3K/PI3K (Fig. 5b), p-AKT/AKT (Fig. 5c) and p-mTOR/mTOR (Fig. 5d) in the sh-COL1A1 group were significantly lower than those in the sh-NC group (P < 0.05). These results suggest that the regulation of the COL1A1 gene on the proliferation and migration of goat granulocytes may be achieved through regulation of the PI3K/AKT/mTOR pathway.

3 Discussion

The extracellular matrix is distributed among cells, especially in connective tissue, where the cell population is relatively sparse and its content is more abundant. The extracellular matrix regulates various cell functions by interacting with cell adhesion molecules, regulators and connexins, such as cell proliferation, differentiation, migration and invasion. Collagen is the main structural protein and plays a dual role in maintaining tissue structure and participating in biological reactions. Studies have shown that the abnormal expression of proteoglycans, laminin and integrin in the extracellular matrix can lead to reduced cell proliferation and then adversely affect tissue development. The COL1A1 gene is an important component of extracellular matrix type I collagen and is closely related to the normal function of human and animal ovaries. In this study, immunohistochemical tests showed that the COL1A1 protein had positive immune responses in the ovarian follicles, ovarian cells and connective tissue of polytocous goats, and the positive area rate, positive cell number and staining intensity of polytocous goats were higher than those of monotocous goats. It is also expressed in the cytoplasm and nucleus of granulosa cells. The same results were obtained by Western blotting, which indicated that the expression of COL1A1 protein in ovarian tissues of polytocous goats was significantly higher than that of monotocous goats, and the ovarian tissues of polytocous goats showed larger morphology and higher follicle maturity. Previous studies have found that abnormal expression of the COL1A1 gene may affect the structure and function of normal ovaries, and its expression level is closely related to several ovulation disorders, such as polycystic ovary syndrome (PCOS). In addition, the abnormal expression of the COL1A1 gene also reflects morphological changes in the structure and organization of the bovine foetal ovary, indicating that the COL1A1 gene plays a role in regulating the development of the bovine foetal ovary and the remodelling of the embryonic mesenchyme. In conclusion, we speculate that the high expression of
COL1A1 protein in ovarian tissue of polytocous goat goats may promote the association between growth factors, cell adhesion molecules, regulatory factors and connexins, thereby strengthening the communication between cells and jointly maintaining ovarian tissue structure and follicle development by providing structural support for cells. This may be an important reason for the different expression levels of COL1A1 protein and the difference in ovarian histomorphology between monotocous and polytocous goats of Guizhou blank goat.

Granulosa cells are important somatic cells in ovarian follicles during follicle development and are related to apoptosis, the cell cycle, cell migration and vessel wall formation. The proliferation of granulosa cells can promote follicle development and maturation, and their apoptosis can trigger follicle atresia. Cell proliferation and apoptosis are complex protein-regulated processes that are related to PCNA, the BCL-2 family, the caspase family and many pathway proteins. Studies have shown that PCNA is involved in various cellular processes, such as DNA replication, DNA repair, chromatin remodelling, and cell cycle control, and is closely related to cell proliferation. In this study, it was found that upregulation of the COL1A1 gene can significantly promote cell proliferation and increase the expression of PCNA protein, while downregulation of the COL1A1 gene has the opposite effect. Since regulation of the cell cycle is crucial for cell proliferation, its different stages provide essential energy, protein, nucleic acid stores, and cell signals for cell division. The study of the granulosa cell cycle showed that upregulation of the COL1A1 gene can promote the development of granulosa cells from G1 phase to G2/M phase, thus promoting the proliferation of granulosa cells. In contrast, the downregulation of COL1A1 expression caused granule cells to stay in G1 phase and reduced the number of organelles, such as mitochondria and ribosomes, that were being prepared for DNA replication and protein synthesis, and the cell proliferation ability was reduced. These results were consistent with the results of the cell proliferation assay. In addition, it was found that the downregulation of COL1A1 inhibited the proliferation of gastric cancer, oral squamous cell carcinoma and bovine cumulus cells. Relevant reports were consistent with the results of this study, indicating that the downregulation of the COL1A1 gene seriously affected the proliferation ability of cells. BAX is a proapoptotic protein that is a major mediator of mitochondria-dependent programmed cell death. Overexpression of BAX can release the activation of caspase-3 protease and mediate cell death. In addition, normal cell metabolism produces low reactive oxygen species (ROS), while excessive ROS can lead to DNA damage, lipid and protein denaturation and decreased mitochondrial activity, which can directly affect caspase function in endogenous and exogenous apoptotic pathways and inhibit cell proliferation, migration and angiogenesis. Therefore, normal ROS levels in cells are critical for granulosa cell proliferation and apoptosis. In this study, by upregulating or downregulating the expression of the COL1A1 gene in ovarian granulosa cells, we found that upregulating the COL1A1 gene could significantly reduce the ROS content in granulosa cells, promote the migration ability of granulosa cells, and inhibit the expression of the proapoptotic proteins BAX, caspase-3 and caspase-9. In contrast, inhibiting COL1A1 expression significantly increased the reactive oxygen species content in granulosa cells, decreased the cell migration ability, and promoted the expression of the BAX, caspase-3 and caspase-9 proteins. These results suggest that the decrease in COL1A1 gene activity leads to an abnormal increase in intracellular
ROS content, mediating mitochondrial dysfunction by promoting the expression of BAX protein and activating the function of caspase family proteins, resulting in cellular inflammation and the induction of granulosa cell apoptosis.

The PI3K/AKT/mTOR pathway, as an important signalling pathway affecting cell survival, proliferation, angiogenesis and migration\textsuperscript{38}, is also a key pathway for promoting ECM synthesis\textsuperscript{39,40}. Studies have found that the activation of the PI3K/AKT/mTOR pathway induces gene translocation into the nucleus, causes the proliferation of Hematopoietic stem cells (HSCs), induces the expression of α-smooth muscle actin (α-SMA) and other cytokines, and generates a large amount of ECM\textsuperscript{41}. Collagen synthesis is also closely related to ovarian development and follicle growth\textsuperscript{18}. In this study, after interfering with and overexpressing the COL1A1 gene, it was found that upregulation of the COL1A1 gene could significantly promote the protein ratios of the p-PI3K/PI3K, p-AKT/AKT and p-mTOR/mTOR pathways and vice versa. Combined with previous studies, the COL1A1 gene is widely and highly expressed in the ovarian tissue of goats, and upregulation of the COL1A1 gene significantly promotes the proliferation and migration of granulocyte cells, indicating that overexpression of the COL1A1 gene could activate the PI3K/AKT/mTOR pathway, promote the synthesis of ECM and regulate the biological effects of granulocyte cells. This is consistent with the findings of Penglei Shen et al. on bovine oocytes\textsuperscript{42}. In addition, Wang found that miR-29 regulated the proliferation of goat ovarian granulosa cells through the PI3K/AKT/mTOR signalling pathway\textsuperscript{43}. Silva's study found that the PI3K pathway acted on ovine ovaries, and gallic acid was used to induce ovine follicle development through PI3K pathway treatment\textsuperscript{44}. In conclusion, the upregulation of the COL1A1 gene can activate the PI3K/AKT/mTOR signalling pathway, promote the synthesis of ECM to regulate the proliferation and migration of granulocytes, and affect the development and maturation of goat follicles.

4 Materials And Methods

4.1 Ethics declarations

The study was conducted according to the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China (No. 588, revised 2017). The use of experimental animals in this study was approved by the Laboratory Animal Ethics of Guizhou University (No. EAE GZU-2021-T083, Guiyang, China; 12 July 2021).

4.2 Animals

Guizhou black goats used in the experiment were provided by the Maiping Black Goat Breeding Base of Guizhou Animal Husbandry and Veterinary Research Institute. The goats were assigned to monotocous and polytocous groups according to their reproductive records, namely the female goats gave birth to more than twin lambs and produced single lamb thought two consecutive cycle time were defined into the monotocous and polytocous groups, respectively. Six monotocous and six polytocous Guizhou black goats (\textit{Capra hircus}) with similar parity, body weight (approximately 30 kg) and without genetic defects in
reproductive organs were selected for simultaneous oestrus treatment. All female goats were raised in same condition. During the first day of oestrus, three goats of each group were hysterectomized after induction of anaesthesia. Fresh ovarian tissues were collected to use for extract tissue protein and immunohistochemistry.

The ovarian granulosa cell preparation procedure followed the method proposed by Zhou et al\textsuperscript{9}, store at -80 degrees Celsius.

### 4.3 Immunohistochemistry

The collected ovarian tissues were fixed with paraformaldehyde for 24 h and then stored at room temperature after paraffin embedding, cooling, sectioning and baking. The sections were successively put into dewaxed solution and graded ethanol for dewaxing and hydration, washed twice with distilled water, and placed in a repair box filled with citrate antigen retrieval buffer (pH 6.0) in a microwave oven for antigen repair. After natural cooling, the slides were placed in PBS (pH 7.4) and washed 3 times with shaking on a decolorization shaker for 5 min each time. Then, the sections were placed in a 3% hydrogen peroxide solution, incubated at room temperature for 25 min in the dark, and washed 3 times for 5 min each time. The tissue was uniformly covered with 3% BSA in the tissue chemical circle, and the tissue was blocked for 30 min at room temperature. After gently shaking off the blocking solution, the rabbit anti-COL1A1 polyclonal antibody (1:100 (volume ratio), PA535380, Thermo Fisher Scientific Co., LTD., Shanghai, China) primary antibody solution prepared in PBS was added to the sections, and the sections were incubated overnight at 4°C in a wet box. The next day, the slides were washed 3 times in PBS with shaking for 5 min each time. After the sections were slightly dried, the tissue was covered by dropping PBS-diluted HRP-labelled secondary antibody (A0453, Beyotime Biotechnology, Shanghai, China) solution, and the sections were incubated at room temperature for 50 min, after which the slides were washed again 3 times for 5 min each time. After the sections were slightly dried, DAB colour solution was added to the ring, and the colour was developed under the microscope for 5–10 minutes (positive immune reactions were brown and yellow). The colour development was terminated by rinsing the sections with tap water. Then, haematoxylin was used for counterstaining for approximately 3 min, after which the slides were washed with tap water, differentiated with haematoxylin differentiation solution for several seconds, washed with tap water and haematoxylin blue returning solution, and washed with running water. Finally, the sections were dehydrated, cleared, and sealed, and the images were observed and recorded under a microscope (80i, Nikon, Japan). The test results were automatically analysed by Aipathwel (Servicebio, Wuhan, China) digital pathological image analysis software.

### 4.4 Immunofluorescence identification

The expression of follicle-stimulating hormone receptor (FSHR) specific protein and the localization of COL1A1 protein in granulosa cells were detected by indirect immunofluorescence. A total of $2 \times 10^5$ granulosa cells were uniformly inoculated into a 35 mm$^2$ culture dish. After 12 hours of culture, the cells were fixed, transparent and sealed, and then added with PBS diluted anti FSHR (1:100; Bios, bs-0895R, Beijing, China) and anti-COL1A1 (1:250; 1:1000; PA5-35380, Thermo Fisher Scientific Co., LTD., Shanghai,
China) primary antibody solution. After incubation overnight at 4°C, the cells were added with PBS diluted Cy3-labeled goat anti-rabbit IgG (H + L) (1:500) solution for 1 hour in dark. Finally, DAPI was added to stain the nuclei for 10 min. Fluorescence microscopy was used to observe and photograph the expression and localization of FSHR and COL1A1 proteins in granulosa cells.

### 4.5 RNA interference and construction of the expression vector

According to the coding region sequence of the goat COL1A1 gene (XM_018064893.1) published in NCBI, the COL1A1 gene interference vector was designed and constructed using free shRNA design software (Clone-Tech Company). Four shRNA interference target sequences with the highest efficiency of the COL1A1 gene were selected according to shRNA design principles, and a pair of negative control sequences were designed to construct the sh-COL1A1 interference vector. The primers were synthesized by Shanghai GenePharma Co., Ltd., and the shRNA that interfered with COL1A1 gene expression with the highest efficiency was screened and verified in the previous stage. Primer sequences are shown in Table 1.

<table>
<thead>
<tr>
<th>Gene name Primer sequence</th>
<th>Primer sequences of COL1A1 interference vector</th>
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<tbody>
<tr>
<td>sh-COL1A1 F: CACCGCAAGGACCTGTCTTTCTAACTTTCAAGAGAGTTAGAAGGACAGGTCTTGTGTTTTTG</td>
<td>sh-COL1A1 R: GATCCAAAGCAGGACTGTCTTCTCTCTCTCGAGTTAGAAGGACAGGTCTTGC</td>
</tr>
<tr>
<td>R: GATCCAAAGCAGGACTGTCTTCTCTCTCTCGAGTTAGAAGGACAGGTCTTGC</td>
<td></td>
</tr>
<tr>
<td>shRNA-NC F: CACCGTTCTCCGAACAGTGCTACGGTTCAAGAGAAGCTGACACGTTCGAGAATTGGG</td>
<td>shRNA-NC R: GATCCAAAAAATTCTCCGAACAGTGCTACGGTTCAAGAGAAGCTGACACGTTCGAGAATTGGG</td>
</tr>
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</table>

Due to the long sequence of the COL1A1 gene, segmented amplification was performed in this experiment. The COL1A1 gene sequence was divided into two fragments for amplification: A (3122 bp) and B (1401 bp). At the same time, different primer sequences were designed, and the A and B fragments were cloned by overlapping extension PCR technology. Then, fragment A was reconstituted into pUC57 (Hind III/BamH), and fragment B was reconstituted into pcDNA3.1(+) (BamH /Not ). After the A and B fragments were correctly linked to the vector, A was digested with Hind III/ BamH I, and B was digested with BamH II /Not I. Then, A and B were linked by T4 DNA ligase to obtain the correct full-length sequence, and the pcDNA3.1(+) -COL1A1 eukaryotic expression vector was constructed.

### 4.6 Cell transfection

Granulosa cells were then removed from the freezer and centrifuged at 1000 rpm for 5 min. Then, the supernatant was discarded, an appropriate amount of medium was added, and a cell suspension was made by blowing and mixing repeatedly. A total of $3 \times 10^5$ cells per well were evenly added to a 6-well plate, and then 2 mL of complete medium was added and mixed by the crossing method. After overnight culture, the medium was replaced with antibiotic-free medium with 3% serum. sh-COL1A1, sh-NC,
pcDNA3.1(+)-COL1A1 and pcDNA3.1(+) no-load plasmids were transfected according to the LipofectamineTM 2000 (11668500, Thermo Fisher Scientific Co., LTD., Shanghai, China) Reagent transfection kit.

4.7 Reactive oxygen detection

A ROS kit was used to detect the intracellular ROS levels in goat granulosa cells. A total of $1 \times 10^4$ granulosa cells were inoculated into 96-well plates. After overnight culture, the cells were transfected with sh-COL1A1, sh-NC, pcDNA3.1(+)-COL1A1 and pcDNA3.1(+) plasmids and cultured for 48 h (5 replicates in each group). Then, the culture medium was discarded, and the cells were washed with PBS three times. A DCFH-DA (2,7-dichlorofluorescin dictate) probe diluted with serum-free culture medium was added to the cells, incubated at 37°C, for 30 minutes, and washed with PBS three times. Finally, a microplate reader was used to measure the fluorescence intensity at an excitation wavelength of 502 nm and an emission wavelength of 530 nm. The fluorescence intensity is proportional to the level of ROS in cells.

4.8 Cell proliferation

A cell counting kit (CCK-8; CA1210, Solarbio) was used to detect the effects of upregulation and downregulation of COL1A1 on the proliferation of granulosa cells. A total of $1 \times 10^4$ cells were evenly seeded in a 96-well plate (5 replicates in each group). After overnight culture, 10% complete medium without serum was replaced, and the cells were transfected with sh-COL1A1, sh-NC, pcDNA3.1(+) -COL1A1 and pcDNA3.1(+) plasmids. The medium was discarded at 0 h, 24 h, 48 h and 72 h, and 10 µL CCK-8 and 90 µL mixed solutions of serum-free medium were added and incubated at 37°C, for 2 h. Finally, the absorbance at 450 nm was measured with a microplate reader (Thermo Fisher Scientific Co., LTD).

4.9 Cell migration

Granulosa cells were seeded in six-well plates and cultured overnight at 37°C. The second day, a 10-µL sterile pipette tip was used to scratch the culture plate. Cells were washed twice with PBS to remove scraped cells. Images were taken under an inverted microscope and recorded as the 0 h timepoint. Then, antibiotic-free medium containing 10% serum was used to transfect sh-COL1A1, sh-NC, pcDNA3.1(+) -COL1A1 and pcDNA3.1(+) plasmids. After continuous cultivation for 24 h, images were taken at the same position and recorded for 24 h.

4.10 Cell cycle

The goat granulosa cell cycle was detected using a cell cycle kit (G1021, Servicebio). Cells were transfected with sh-COL1A1, sh-NC, pcDNA3.1(+) -COL1A1 and pcDNA3.1(+) plasmids for 48 h. The treated cells were harvested, washed twice with PBS, resuspended by slowly adding 1 mL precooled 75% ethanol to the cell precipitate, and then fixed overnight at 4°C. On the second day, the cells were collected by centrifugation and washed once with PBS. Then, the cells were resuspended by adding 100 µL PBS and 2 µL RNase A (10 mg/mL) and incubated in a 37°C water bath for 30 min, after which RNA was digested, followed by the addition of 100 µL propidium iodide (PI) (100 µg/mL) for staining for 10 min,
avoiding light. Finally, the stained cells were detected by flow cytometry (BD Biosciences). Data were analysed using Modfit software to determine the cell cycle distribution.

4.11 Western blotting

The fresh ovarian tissue was cut into pieces and then homogenized with 1 mL RIPA (P0013B, Beyotime Biotechnology) lysate buffer (containing 10% PMSF), and the tissues were fully lysed on ice for 20 min. The supernatant was centrifuged, and the total tissue protein was extracted. After transfection for 48 h, RIPA lysis buffer (containing 10% PMSF) was added, and the cells were lysed fully at 4°C for 20 min. The supernatant was collected by centrifugation, and the total cell protein was extracted. Then, a BCA protein assay kit (PC0020, Solarbio, Beijing, China) was used to determine the protein concentration. The protein samples were diluted to the same concentration and then mixed with protein loading buffer. The samples were denatured at 100 °C for 10 min and then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; P0012A, Beyotime Biotechnology). After the proteins were transferred to PVDF membranes and blocked, the membranes were placed in TBST diluted anti-COL1A1 (1:1000; PA5-35380, Thermo Fisher Scientific Co., LTD., Shanghai, China), anti-p-PI3K (1:1000; AF3241, Affinity Biosciences, Jiangsu, China), anti-p-MTOR (1:500; YP0176, ImmunoWay Biotechnology, USA), anti-caspase-3 (1:1000; 9668S, Cell Signaling Technology, Carlsbad, CA, USA), anti-p-AKT (1:2000; 4060S, Cell Signaling Technology), anti-caspase-9 (1:2000; AB202068, Abcam, USA), anti-GAPDH (1:5000; ABS132004, Absin Biotechnology Co., LTD, Beijing, China), anti-BAX (1:1000; 505992-1-lg, Proteintech, Wuhan, China), anti-PCNA (1:1000; 60097-1-lg, Proteintech), anti-AKT (1:2000; 60203-1-lg, Proteintech), anti-PI3K (1:1000; 67121-1-lg, Proteintech), and anti-mTOR (1:5000; 66888-1-lg, Proteintech) solutions and were incubated overnight at 4 °C and then washed with 1×TBST 3 times. Then, the PVDF membranes were placed into goat anti-rabbit or goat anti-mouse secondary antibody solution, incubated at 37 °C for 1.5 h and washed with 1× TBST 3 times. Finally, enhanced chemiluminescence (ECL; P0018AS, Beyotime Biotechnology) hypersensitive chemiluminescence solution was added for colour development, and then a gel imaging system was used for imaging analysis. ImageJ V1.8.0 software (National Institutes of Health) was used for densitometric analysis.

4.12 Statistical analysis

SPSS 19.0 software was used to analyse the differences in cell proliferation, cell migration, and protein expression levels between the sh-COL1A1, sh-NC, pcDNA3.1(+)-COL1A1 and pcDNA3.1(+) groups. A t test was used to evaluate the difference in COL1A1 protein expression between monotocous and polytocous ovaries, and GraphPad Prism 5 graphic analysis software (GraphPad Software, Inc.) to create graphs. All experiments were repeated more than 3 times, and the data represent the mean ± SD.

5 Conclusions

In this study, our data indicate that COL1A1 had a higher expression level in ovarian tissues of the polytocous group compared with the monotocous group. Upregulated COL1A1 may facilitate follicular growth and maturity by promoting granulosa cell proliferation and migration and activating the
PI3K/AKT/mTOR signalling pathway, which is conducive to the synthesis and distribution of ECM in ovarian tissues and reduces ROS production in granulosa cells. Hence, COL1A1 could be a vital molecule in follicular development that further influences reproductive performance in goats.

**Declarations**

We confirm that this study is based on ARRIVE guidelines https://arriveguidelines.org Reporting.

**Competing interests:** The author(s) declare no competing interests.

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**Author Contributions statement:** Mingshuai Zhou and JiaFu Zhao conceived and designed the experimental program. Xiaoyan Wen, Qingmei Lu, Bin Liu, Yang Yang and Chao Yuan assisted in the collection of tissues and cells of experimental animals. Mingshuai Zhou performed the experiments. Mingshuai Zhou, Jiafu Zhao, Xiang Chen and Zheng Ao wrote and edited the manuscript and take responsibility for the integrity and security of the data.

**Data Availability:** The data presented in this study are available on request from the corresponding author.

**References**


**Figures**
Figure 1

Expression and localization of COL1A1 protein in ovarian tissues of monotocous and polytocous goats. (a) Immunohistochemical results of ovarian tissue of monotocous goats and polytocous goats. Granulosa and theca cells in the mature follicle, Connective tissue and Primordial follicle. Scale bar=100 μm. Secondary follicles. Scale bar=20 μm. (b) Western blotting was used to detect the expression of COL1A1 in ovarian tissues of monotocous and polytocous goats. (c) The results of quantitative analysis
of the protein bands of COL1A1. (d) H-Score = The number of positive cells and their staining intensity. (e) External morphological observation of ovarian tissue of monotocous goats and polytocous goats. (f) Positive area ratio = Positive area/tissue area. *(P<0.05) **(P<0.01)

Figure 2

The expression of FSHR and COL1A1 protein in cells was detected by indirect immunofluorescence.

Figure 3
Effects of different COL1A1 expression levels on granulosa cell migration and ROS levels. (a) The expression levels of COL1A1 were detected by Western blotting 48 hours after transfection. (b) The results of quantitative analysis of the protein bands of COL1A1. (c) Effect of the COL1A1 gene on ROS levels in granulosa cells. (d) Effects of different expression levels of COL1A1 gene on granular cell migration. (e) Migration distance of granulosa cells in different transfection groups. with different capital letters indicating significant differences ($P<0.05$) and the same letter indicating no significant differences ($P>0.05$).

**Figure 4**

Effects of different expression levels of COL1A1 on granulosa cell proliferation and cell cycle. (a) The expression levels of PCNA, BAX, caspase-3 and caspase-9 were detected by Western blotting 48 hours after transfection. (b-e) The results of quantitative analysis of the protein bands of PCNA(b), BAX(c),...
caspase-3(d) and caspase-9(e). (f) The effects of sh-COL1A1, sh-NC, pcDNA3.1(+)-COL1A1 and pcDNA3.1(+) on the proliferation of granulosa cells were determined by CCK8. (g) The effects of sh-COL1A1, sh-NC, pcDNA3.1(+)-COL1A1 and pcDNA3.1(+) on granulosa cell cycle were analyzed by flow cytometry. The results were analysed using multiple comparisons and t test, with different capital letters indicating significant differences ($P<0.05$) and the same letter indicating no significant differences ($P>0.05$). *(P<0.05)** *(P<0.01)*

Figure 5

Effects of different COL1A1 gene expression levels on the p-PI3K/PI3K, p-AKT/AKT and P-mTOR/mTOR ratios. (a) Western blotting was used to detect the expression levels of p-PI3K, PI3K, p-AKT, AKT, p-mTOR and mTOR in sh-COL1A1, sh-NC, pcDNA3.1(+)-COL1A1 and pcDNA3.1(+). (b-d) Effects of COL1A1 gene expression on p-PI3K/PI3K(b), p-AKT/AKT(c), and p-mTOR/mTOR(d) protein expression. The results were
analysed using multiple comparisons, with different capital letters indicating significant differences ($P<0.05$) and the same letter indicating no significant differences ($P>0.05$)