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Research Article

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Cathepsin D knockdown regulates biological behaviors of granulosa cells and affects litter size traits in goats

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Abstract: Cathepsin D (CTSD), the major lysosomal aspartic protease that is widely expressed in different tissues, potentially regulates the biological behaviors of various cells. Follicular granulosa cells are responsive to the increase of ovulation number, hence indirectly influencing litter size. However, the mechanism underlying the effect of CTSD on the behaviors of goat granulosa cells has not been fully elucidated. This study used immunohistochemistry to analyze CTSD localization in goat ovarian tissues. Moreover, western blotting was applied to examine the differential expression of CTSD in the ovarian tissues of monotocous and polytocous goats. Subsequently, the effects of CTSD knockdown on cell proliferation, apoptosis, cell cycle, and the expression of candidate genes of the prolific traits, including bone morphogenetic protein receptor IB (*BMPR-IB*), follicle-stimulating hormone (*FSHR*), and inhibin α (*INHA*), were determined in granulosa cells. Results showed that CTSD was expressed in corpus luteum, follicle, and granulosa cells. Notably, CTSD expression in the monotocous group was significantly higher than that in the polytocous group. In addition, CTSD knockdown could improve granulosa cell proliferation, inhibit cell apoptosis, and significantly elevate the expression of proliferating cell nuclear antigen (*PCNA*) and B cell lymphoma 2 (*Bcl-2*), but it lowered the expression of Bcl-2-associated X (*Bax*) and *caspase-3*. Furthermore, CTSD knockdown significantly reduced the ratios of cells in the G0/G1 and G2/M phases but substantially increased the ratio of cells in the S phase. The expression levels of *cyclin D2* and *cyclin E* were elevated followed by the obvious decline of *cyclin A1* expression. However, the expression levels of BMPR-IB, FSHR, and INHA clearly increased as a result of CTSD knockdown. Hence, our findings demonstrate that CTSD is an important factor affecting the litter size trait in goats by regulating the granulosa cell proliferation, apoptosis, cell cycle, and the expression of candidate genes of the prolific trait.

Key words: Cathepsin D (CTSD); Litter size trait; Granulosa cells; Cell apoptosis; Cell cycle; Cell proliferation

1 Introduction

Multiple factors co-regulate the reproductive capacity of goats; however, the reproductive characteristics of goat breeds or lines directly affect their reproductive phenotype and gene selection bias (Lv et al., 2014; Bowman et al., 2018). Of note, the "Qianbei Ma" goat is a fine local breed of Guizhou Province in China, with a litter size rate up to 196%. It exhibits early sexual maturity and year-round estrus cycle; thus it is considered as an ideal model for studying the reproductive traits of goats (Cai et al., 2014; Sui et al., 2015).

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Litter size is a critical goat reproductive trait, and the ovary plays a vital role in lambing. Moreover, genetic differences of the ovary play a crucial role in variations in follicle development, ovulation, the secretion of female hormones, and litter size (Soede et al., 2011; Pan et al., 2012; Hsueh et al., 2015). Therefore, the appropriate development of the ovary is essential for the reproductive capability of mammals, including goats. The follicle, which is the basic unit driving ovarian function, including growth and development (McGee and Hsueh, 2000), continuously matures until ovulation. Its growth, development, and survival are regulated by multiple secretory hormones and growth factors (Gougeon, 1996; Martins et al., 2014). For instance, granulosa cells have been shown to control follicular growth, development, and atresia by regulating proliferation, differentiation, cell cycle, and apoptosis (Kranc et al., 2017). Previously, different

studies established the presence of bone morphogenetic protein receptor IB (BMPR-IB), follicle-stimulating hormone (FSHR) and inhibin α (INHA) in granulosa cells and associated them with prolific goat traits and ovulation number (Bradford, 1972; Mahdavi et al., 2014; Cui et al., 2019; Xia et al., 2020). Notably, ovulation is also related to tissue remodeling; the follicular theca fuses with the tunica albuginea, and then thins. Afterwards, the rupture of follicle is triggered by a proteolytic enzyme in follicular fluid, which induces ovulation (Boots and Jungheim, 2015).

The lysosome contains proteolytic enzymes, such as cathepsin D (CTSD), which is a member of the lysosomal aspartic protease family. Aspartic protease is a proteolytic enzyme in the pepsin family and is a glycoprotein. CTSD was investigated in depth and compared to other aspartic proteases (Dubey and Luqman, 2017). It was also reported as an important regulatory factor affecting the different stages of endogenous and exogenous cell apoptosis pathways (Minarowska et al., 2007). CTSD induces cell apoptosis under cytotoxic conditions. Besides, it is involved in mitosis, which prompts the activation of leukocytes and regulates vascular wall permeability (Minarowska et al., 2007). Moreover, the proteolytic activity of this enzyme is regulated by various factors in the lysosome, including pH value, metabolites, hormones, growth factors, and specific inhibitors (Liu et al., 2016; Francisco et al., 2019). In addition, CTSD exerts a significant role in the hydrolysis and conversion of various proteins in cell processes, and it is the ubiquitous lysosomal endoprotease of mammalian cells (Minarowska et al., 2007). Simultaneously, CTSD also functions in protein degradation (Ge et al., 2018). In fish, CTSD mediates the processing of vitellin in the oocyte; therefore, it is essential for living egg production (Brooks et al., 1997). Brooks et al. (1997) have reported that CTSD messenger RNA (mRNA) is expressed in both ovarian and non-ovarian tissues (including liver, muscle, spleen, and testicles). During ovarian development, the highest expression level of CTSD mRNA occurs at the early stage of vitellogenesis, and then this level gradually declines (de Stasio et al., 1999). Thus, it was suggested that CTSD contributes to the comprehensive growth and development of ovarian tissue, and has an essential function in determining animal reproductive traits (de Stasio et al., 1999). A recent sequencing study identified the differential expression of CTSD in the ovaries of monotocous and polytocous sheep (Feng et al., 2018). Our previous transcriptome sequencing findings showed that, in comparison with monotocous goats, CTSD expression was significantly down-regulated in the ovarian tissues of polytocous goats (unpublished data). Therefore, CTSD regulates follicular development and biological function in goat granulosa cells. However, no study has yet evaluated the effect of CTSD on the biological behavior of goat granulosa cells or the litter size trait.

In this work, we (1) explored the correlation between CTSD expression and litter size in goats and the effects of CTSD on the biological behavior of granulosa cells, as well as the location of CTSD in goat ovarian tissue; (2) analyzed the differential expression of CTSD in ovarian tissues between monotocous and polytocous "Qianbei Ma" goats; and (3) revealed the effects of CTSD knockdown on cell proliferation, apoptosis, cell cycle, and the expression of candidate genes of the prolific trait in granulosa cells.

2 Materials and methods

2.1 Source of experimental animals

Ten "Qianbei Ma" goats (aged 36 months) with an initial average body weight of (38.6±2.8) kg (mean±standard deviation (SD)) were obtained for the experiment. Ewes with two consecutive litter size records were divided into two groups: a polytocous group (n=5; litter size=3) and a monotocous group (n=5; litter size=1). Both groups were housed under conditions of constant temperature (25 °C) and fixed light/dark cycle (12 h/12 h) at the site of Fuxing Husbandry Co., Ltd. (Guizhou, China) and were provided with feed and water ad libitum. The housing had a high "bed" fence and all goats were clinically healthy. Before the beginning of the experiment, estrus was synchronously inducted in ewes. Then, a sponge suppository was implanted into the ewes' vagina for 13 d, after which the animals were injected 0.5 mg cloprostenol, and the sponge was retrieved. Two days after injection, the ewes were checked for signs of estrus at 10:00 am and 4:00 pm. After confirming the presence of estrus, the ewes were individually slaughtered, and ovarian samples were collected and immediately preserved in liquid nitrogen awaiting RNA and protein extraction. Furthermore, the right

ovaries of polytocous "Qianbei Ma" goats were collected and washed with sterile phosphate-buffered saline (PBS) containing antibiotics (penicillin and streptomycin) for further storage and the isolation of follicular granulosa cells (>2.5 mm). Subsequently, the right ovarian tissues used for immunohistochemistry were fixed in a 4% (volume fraction) paraformaldehyde (PFA) solution and paraffin-embedded.

2.2 Immunohistochemistry

The immunohistochemistry protocol was performed according to that described previously (Peng et al., 2016). In short, paraffin sections with 5-μm thickness were attached to microscope slides, heated at 65 ℃ for 2 h, dewaxed in xylene, and then rehydrated in a graded series of ethanol. Subsequently, these sections were placed in 0.1 mol/L sodium citrate buffer (pH 6.0), boiled in the microwave for 15 min, and then cooled to 37 ℃. After rinsing with PBS, the activity of endogenous peroxidase was blocked by immersing the sections in 3% (mass fraction) hydrogen peroxide (H_2O_2) for 15 min. To block the non-specific staining process, the sections were immersed in a blocking solution (5% (mass fraction) bull serum albumin (BSA) in TBSTw (including Tris-HCl, NaCl, and Tween 20)) at 37 ℃ for 15 min. The sections were then immersed for 12 h at 4 ℃ with rabbit anti-CTSD polyclonal antibody (1:2000 (volume ratio, the same below), Bioss, bs-1500R, Beijing, China) in TBSTw. After rinsing with PBS, the sections were immersed with horse radish peroxidase (HRP)-labelled secondary antibody (A0208, Beyotime Biotechnology, Shanghai, China) at 37 ℃ for 2 h and then with diaminobenzidine for 15 min. The primary antibody was replaced with non-specific rabbit immunoglobulin G (IgG) to act as a negative control (NC). Digital images of immunohistochemically stained sections were captured through three light source microscopes (80i, Nikon, Japan).

2.3 RNA interference

In order to down-regulate CTSD expression, we used four CTSD short hairpin RNAs (shRNAs), including sh1-CTSD, sh2-CTSD, sh3-CTSD, and sh4-CTSD, and negative shRNA control (sh-NC), which were designed and synthesized by GenePharma (Shanghai, China). The information of sh-CTSD and sh-NC fragments is shown in Table 1. Subsequently, we extracted the sh-CTSD plasmids and the corresponding control plasmid (TIANGEN Biotech, Beijing, China) and stored them at −20 ℃ until transfection. We verified the suppression efficiency of the CTSD gene at the transcription and translation levels.

2.4 Cell culture and plasmid transfection

The preparation procedure followed the method proposed by Peng et al. (2016). The ovarian tissue was placed on ultra-clean workbench. Then, we pierced the follicle with a 10-gauge needle, which permitted follicular fluid flow into Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12). This was followed by 10-min centrifugation at 1000 r/min. After discarding the supernatant, the pellet was mixed with DMEM/F-12 medium by pipetting and transferred to a cell culture flask for culture (37 ℃, 5% CO_2). Subsequently, Lipofectamine™ 2000 (Thermo Fisher Technology, Massachusetts, USA) was used to transiently transfect granulosa cells with 15 μg sh1-CTSD to sh4-CTSD or sh-NC. Cells were harvested 48 h post-transfection (Zhang et al., 2018).

Table 1 Information of shRNA fragments

Fragment	Fragment sequences (5'→3')
sh1-CTSD	Forward: CACCGGATCCCACTGCAGAAGTTCATTCAAGAGATGAACTTCTGCAGTGGGATCCTTTTTTG
	Reverse: GATCCAAAAAAGGATCCCACTGCAGAAGTTCATCTCTTGAATGAACTTCTGCAGTGGGATCC
sh2-CTSD	Forward: CACCGCTCAAGAACTACATGGACGCTTCAAGAGAGCGTCCATGTAGTTCTTGAGCTTTTTTG
	Reverse: GATCCAAAAAAGCTCAAGAACTACATGGACGCTCTCTTGAAGCGTCCATGTAGTTCTTGAGC
sh3-CTSD	Forward: CACCGGGTCCACCACAAATACAACATTCAAGAGATGTTGTATTTGTGGTGGACCCTTTTTTG
	Reverse: GATCCAAAAAAGGGTCCACCACAAATACAACATCTCTTGAATGTTGTATTTGTGGTGGACCC
sh4-CTSD	Forward: CACCGCCTGTCTTCGACAACCTGATTTCAAGAGAATCAGGTTGTCGAAGACAGGCTTTTTTG
	Reverse: GATCCAAAAAAGCCTGTCTTCGACAACCTGATTCTCTTGAAATCAGGTTGTCGAAGACAGGC
sh-NC	Forward: CACCGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTTG
	Reverse: GATCCAAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAAC

shRNA: short hairpin RNA; CTSD: cathepsin D; sh-CTSD: CTSD shRNA; sh-NC: negative shRNA control.

2.5 RNA extraction and qRT-PCR analysis

TRIzol® reagent (Invitrogen, Grand Island, USA) was used for total RNA extraction from cultured follicular granulosa cells. Exactly 500 ng of total RNA was obtained for complementary DNA (cDNA) first-strand synthesis by the StarScript II First-strand cDNA Kit (GenStar, Beijing, China). We performed quantitative real-time polymerase chain reaction (qRT-PCR) based on the SYBR fluorescent dye method to quantify CTSD mRNA abundance in follicular granulosa cells (GenStar). A list of the PCR primers used is provided in Table 2. For all reactions, a 20-μL final volume of reaction mixture was prepared, comprising of 10 μL 2× SYBR mixture, 100 nmol/L of each primer, and 1 μL cDNA. The cycling parameters were as follows: one cycle at 95 ℃ for 2 min, followed by 40 cycles at 95 ℃ for 15 s, 60 ℃ for 30 s, and 72 ℃ for 30 s, extended by one cycle at 72 ℃ for 5 min.

2.6 Western blotting

Based on a previously described method (Ao et al., 2019), follicular granulosa cells grown to 90% confluency and ovarian tissues were lysed with radio immunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, Shanghai, China). The concentration of extracted total protein was determined by the BCA protein assay kit (Beyotime Biotechnology). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transmembrane electrophoresis were performed with the Mini-PROTEAN Tube Cell Instrument (Bio-Rad, Hercules, CA). The membranes were incubated with primary antibodies, including anti-CTSD (1:2000; Bioss, bs-1615R, Beijing, China), anti-BMPR-IB (1:1500; Bioss, bs-6639R), anti-FSHR (1:1500; Bioss, bs-0895R), anti-INHA (1:1000, Bioss, bs-1032R), and anti-β-actin (1:3000; Affinity Biosciences, AF7018, USA) (Tang et al., 2019). Next, the membranes were subjected to further incubation with HRP-conjugated goat anti-rabbit secondary antibody (A0208, Beyotime Biotechnology). The immunoreactive bands were visualized using an electrochemiluminescence (ECL) kit (Beyotime Biotechnology). The membrane images were documented using a chemiluminescence imaging system (Bio-Bad, USA), whereas ImageJ (V1.48) was adopted to quantify the densitometric

Table 2 Real-time PCR primer details

Gene	Primer sequence (5'→3')	Product (bp)	Amplification efficiency (%)	GenBank ID
CTSD	Forward: TGACTCGCATGTAGGTTGC	185	98.15	XM_018043344.1
	Reverse: TGGAGACGCTGTAGGAA			
BMPR-IB	Forward: GCTCTTGGTCCTCATCATTTTATTC	99	100.87	XM_013964509.2
	Reverse: ATGTAAGTTTCGTCCTGTTCTAACC			
INHA	Forward: GACCAAGATGTCTCCCAG	166	98.98	NM_001285606.1
	Reverse: CAGTATGGAACCACAGCT			
FSHR	Forward: TGTTATGTCCCTCCTTGTGCTC	129	101.15	NM_001285636.1
	Reverse: CGCTTGGCTATCTTGGTGTCA			
Bcl-2	Forward: TCTTTGAGTTCGGAGGGGTC	188	102.39	XM_018039337.1
	Reverse: TGGTGGAAGAGTGTGCTGTG			
Bax	Forward: CATCGGAGATGAATTGGACAGTAA	178	100.26	XM_013971446.2
	Reverse: GGCCTTGAGCACCAGTTTGC			
caspase-3	Forward: CCTGGACTGTGGTATTGAGA	218	99.12	XM_018041755.1
	Reverse: TAACCCGAGTAAGAATGTGC			
cyclin A1	Forward: GACAGTGCCAACTACCAACC	131	96.77	XM_018056659.1
	Reverse: GGTATTTCAAGAACGGGTCA			
cyclin D2	Forward: GAGCAGAAGTGCGAAGAGGAGG	191	104.54	XM_005680985.3
	Reverse: TTGATGGAGTTGTCGGTGTAAATG			
cyclin E	Forward: GATGTCGGCTGCTTAGAAT	104	103.31	XM_018062248.1
	Reverse: CACCACTGATACCCTGAAAC			
PCNA	Forward: GAAGAAAGTGCTGGAGGC	129	99.93	XM_005688167.3
	Reverse: TCGGAGCGAAGGGTTA			
β-actin	Forward: AGATGTGGATCAGCAAGCAG	139	97.23	NM_001314342.1
	Reverse: CCAATCTCATCTCGTTTTCTG			

CTSD: cathepsin D; *BMPR-IB*: bone morphogenetic protein receptor IB; *INHA*: inhibin α; *FSHR*: follicle-stimulating hormone; *Bcl-2:* B cell lymphoma 2; *Bax*: Bcl-2-associated X; *PCNA*: proliferating cell nuclear antigen; PCR: polymerase chain reaction.

analysis result for the bands. All blots were normalized to β-actin to assess the relative expression of target proteins.

2.7 Cell proliferation

Using the cell counting kit-8 (CCK-8; Beyotime Biotechnology), the proliferation of granulosa cells was evaluated after CTSD knockdown (Zhang et al., 2019). Granulosa cells transfected with sh-NC or sh-CTSD were seeded into 96-well plates and incubated for increasing durations (0, 6, 12, 24, 48, and 72 h) before adding CCK-8 solution (10 μL) into each well for 3 h. Absorbance measurements (at 450 nm) were taken using a microplate reader (Thermo Fisher Technology). Data for the CCK-8 assays were evaluated using a Student's *t*-test, and were presented as mean±SD. $P<0.05$ was the significance threshold.

2.8 Cell apoptosis and cell cycle

The cell apoptosis analysis kit (C1052, Beyotime Biotechnology) was used to detect apoptosis while rigorously following the manufacturer's instructions. Briefly, the harvested cells were washed with PBS, and then resuspended in 500 mL annexin-binding buffer. Annexin V-fluoresceine isothiocyanate (FITC) (4 mL) and propidium iodide (pI) (8 mL) were subsequently added, followed by 5 min incubation at room temperature in the dark. Cells were then subjected to flow cytometry and analyzed by FlowJo software (FACS Calibur, Becton Dickinson, CA, USA).

Moreover, the goat granulosa cells (1×10^5 cells/mL) were detected using a cell cycle kit (KGA512, KeyGEN Biotech, Jiangsu, China). These cells were transfected with sh-NC or sh-CTSD for 48 h. The treated cells were harvested, washed with PBS, and then fixed in 75% (volume fraction) ethanol at 4 ℃ for 16 h. Following centrifugation to remove residual ethanol, cells were washed once with PBS, and incubated in 2 mL staining buffer (A) and 10 mL of reagent B for 20 min at room temperature in the dark. The stained cells were examined using a flow cytometer (Guava easyCyte 6-2L; Millipore, USA). Data were analyzed using the ModFit LT 5.0 software (Verity Software House, USA).

2.9 Statistical analysis

SPSS 19.0 software was employed to analyze the differences in cell proliferation, apoptosis, and cycle data between the sh-CTSD group and the sh-NC group. A Student' s *t*-test was used to evaluate the differences in CTSD protein expression levels between the monotocous and polytocous ovaries. All data represent the mean±SD. The significance threshold was set as $P<0.05$. In this study, three biological repeats and three technical repeats were performed to ensure the accuracy of experimental data.

3 Results

3.1 Location of CTSD in goat ovarian tissues

Immunohistochemistry analysis was performed to determine the expression pattern and distribution of CTSD in ovarian tissues. CTSD was revealed to exist in oocytes from all levels of follicles (Figs. 1a–1e). Additionally, the immunoreaction of CTSD protein was found in corpus luteum and follicular granulosa cells. For all the detected antibodies, the reaction of control group (Fig. 1f) confirmed the nonspecific staining process.

3.2 Expression of CTSD in goat ovarian tissues

Western blotting was employed to confirm the differential expression of CTSD in ovarian tissues of goats with different litter sizes, i.e., to compare CTSD expression in the ovary of monotocous and polytocous goats. We found greater CTSD protein expression in the ovary of monotocous goats than in the ovary of polytocous goats ($P<0.01$; Fig. 2).

3.3 Suppression of CTSD and expression of candidate genes of the prolific trait in goats

To elucidate the influence of CTSD on the goat litter size trait, CTSD expression in granulosa cells was decreased through transfection with specific shRNA. We estimated the changes of candidate genes of the prolific trait in granulosa cells, including *BMPR-IB*, *FSHR*, and *INHA*. Results revealed that, among the four oligonucleotide sequences of sh-CTSD, sh3-CTSD produced the most optimal interference effect on CTSD at the transcriptional level (Fig. 3a). Simultaneously, it could significantly ($P<0.01$) inhibit the expression of CTSD protein at the translational level (Fig. 3c). In addition, the downregulation of CTSD expression in granulosa cells could significantly improve the expression of BMPR-IB, FSHR, and INHA at both

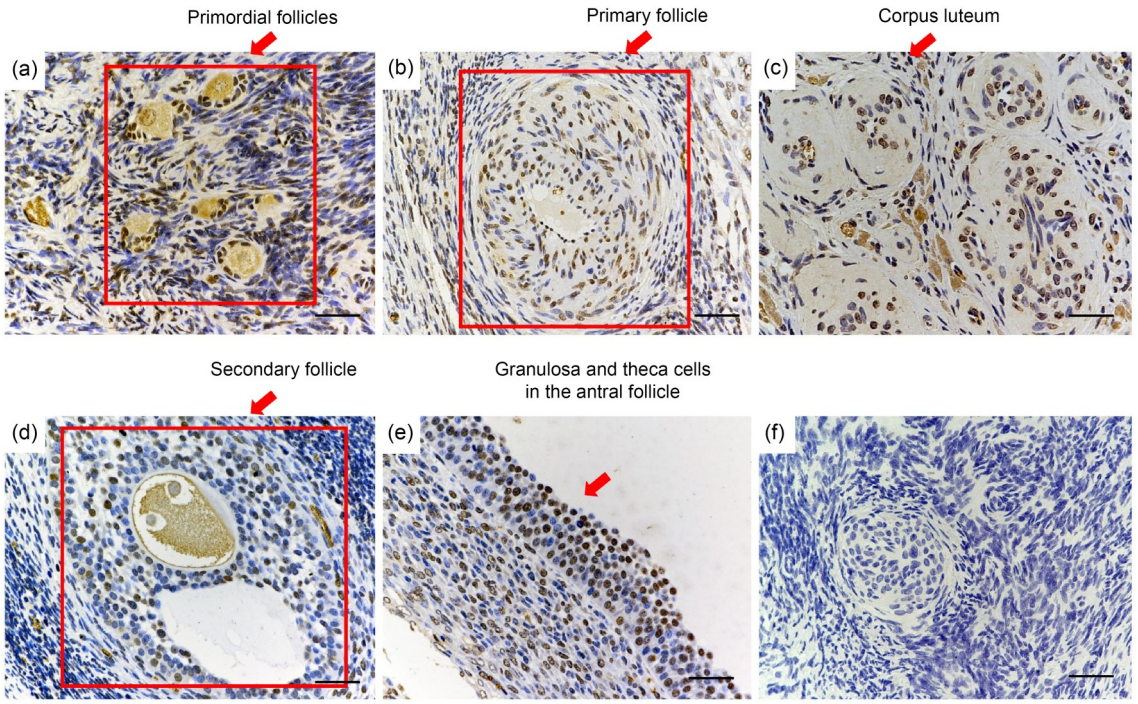


Fig. 1 Analysis of cathepsin D (CTSD) localization at the ovary interface of "Qianbei Ma" goat. Sections of ovarian tissue were prepared and stained for CTSD with a nonspecific immunoglobulin G (IgG) as negative staining control. (a) Primordial follicles; (b) Primary follicle; (c) Corpus luteum; (d) Secondary follicle; (e) Granulosa and theca cells in the antral follicle; (f) Negative control. Scale bar=400 μm.

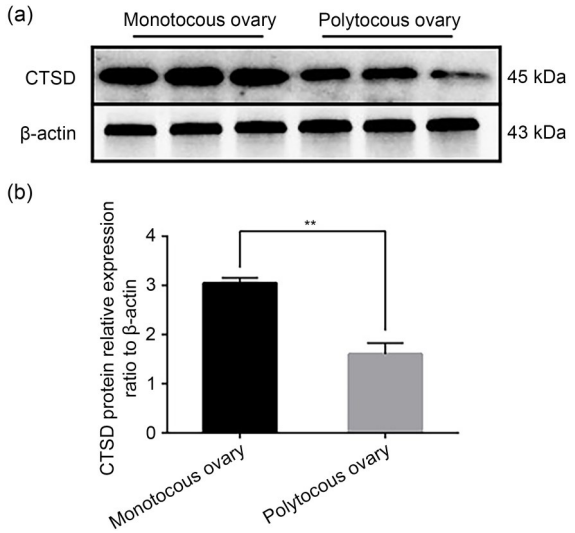


Fig. 2 Expression of cathepsin D (CTSD) in the ovarian tissues of monotocous and polytocous goats. (a) Western blotting was used to analyze CTSD expression in monotocous and polytocous ovaries. (b) Quantification of the CTSD expression level in monotocous and polytocous ovaries, normalized to β-actin expression. Data are presented as mean±standard deviation (SD), n=3. ** P<0.01.

transcriptional and translational levels (Figs. 3b and 3d).

3.4 Inhibition of CTSD, cell proliferation and apoptosis

In order to investigate the effects of CTSD on the biological behavior of follicular granulosa cells, and its expression levels of related genes, including proliferating cell nuclear antigen (*PCNA*), B cell lymphoma 2 (*Bcl-2*), Bcl-2-associated X (*Bax*), and *caspase-3*, specific shRNA was adopted to knock down CTSD expression in granulosa cells. The impacts of CTSD expression on granulosa cell proliferation and apoptosis were evaluated via the combination of CCK-8 colorimetry and flow cytometry. Compared to the granulosa cells treated with sh-NC, the proliferation activity of granulosa cells was increased after the knockdown of CTSD expression. Besides, at 12, 24, 48, and 72 h after shRNA transfection, CTSD knockdown significantly improved the proliferation of granulosa cells (Fig. 4a) and increased the expression levels of *PCNA* genes (Fig. 4b). Compared with sh-NC, sh3-CTSD demonstrated a stronger inhibition effect on granulosa cell apoptosis after transfection (P<0.01; Fig. 4c), wherein the apoptosis rate was decreased by 1.3% (Fig. 4d) and the level of cell anti-apoptotic factor *Bcl-2*

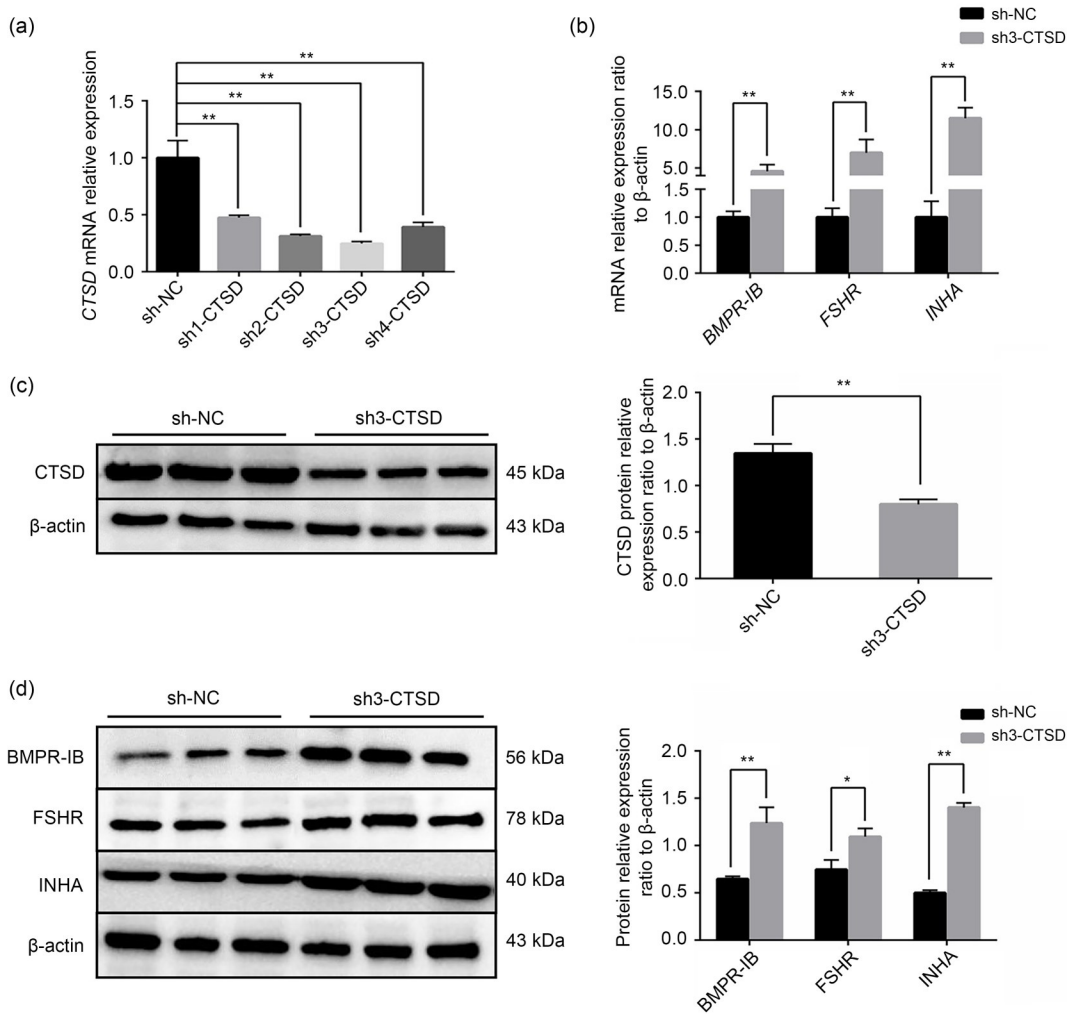
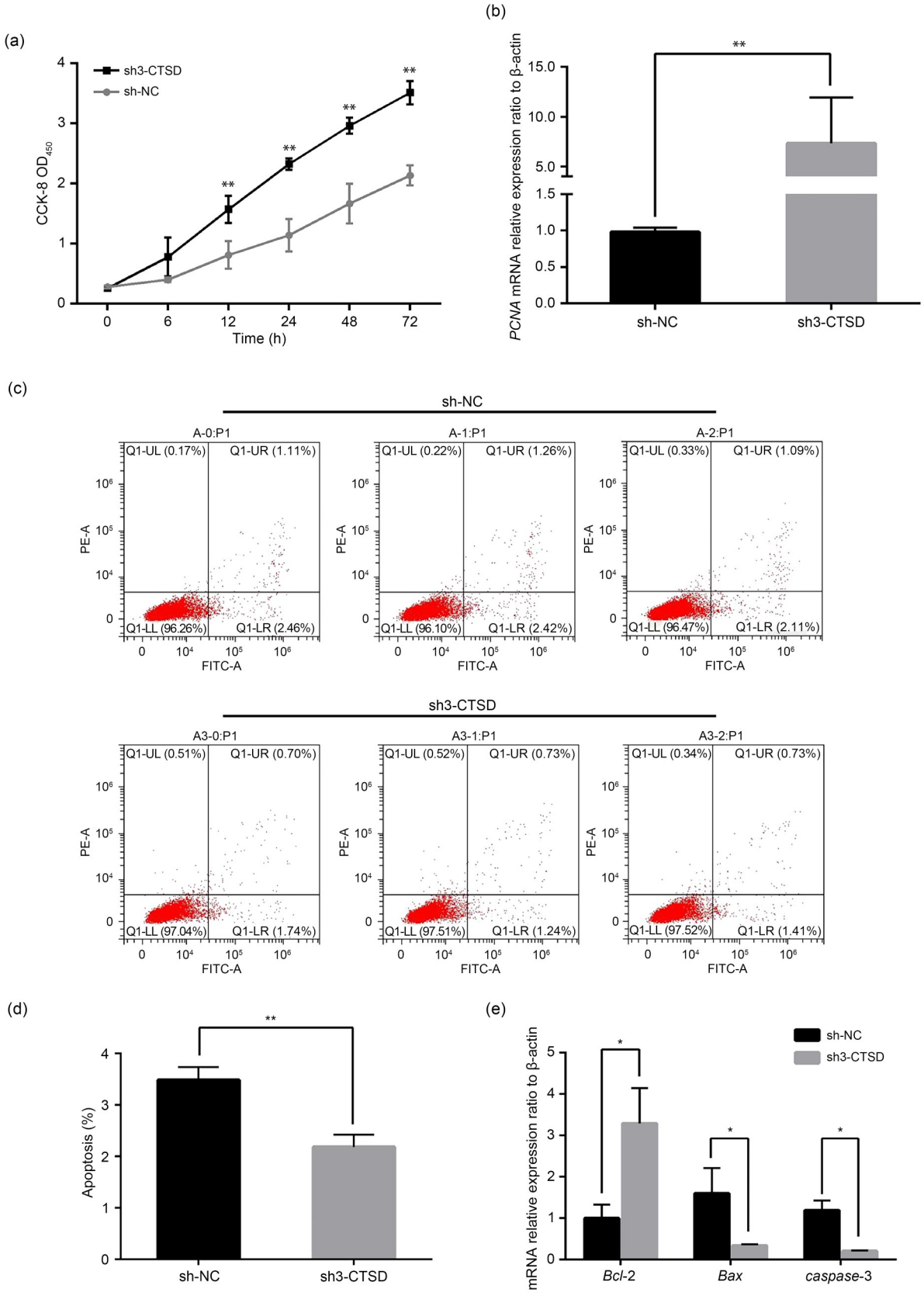


Fig. 3 Effects of cathepsin D (CTSD) knockdown on the expression of BMPR-IB, FSHR, and INHA in granulosa cells. (a) Interfering efficiency of CTSD short hairpin RNA (shRNA) following quantitative real-time polymerase chain reaction (qRT-PCR) analysis; (b) Effect of decreased CTSD on the messenger RNA (mRNA) abundance of bone morphogenetic protein receptor IB (*BMPR-IB*), follicle-stimulating hormone (*FSHR*), and inhibin α (*INHA*) in granulosa cells; (c) Interfering efficiency of CTSD shRNA as revealed by western blotting; (d) Effect of CTSD knockdown on the expression of BMPR-IB, FSHR, and INHA in granulosa cells. Data are presented as mean±standard deviation (SD), n=3. * P<0.05, ** P<0.01.

was significantly higher in granulosa cells after CTSD knockdown. Meanwhile, the expression levels of cell pro-apoptotic factors, *Bax* and *caspase-3*, were obviously reduced (P<0.05; Fig. 4e).

3.5 Inhibition of CTSD on the cycle of follicular granulosa cells

The effects of CTSD knockdown on follicular granulosa cell cycle were explored through annexin-V and PI staining of the granulosa cells treated with sh3-CTSD and subsequent detection by flow cytometry. We found that CTSD knockdown could significantly lower the ratio of granulosa cells in the G0/G1 and G2/M phases (P<0.05), whereas the ratio of cells in the S phase was obviously increased (P<0.01) (Figs. 4f and 4g). In addition, to confirm the effect of CTSD on granulosa cell cycle-related factors, the in vitro expression levels of cyclin A1, cyclin D2, and cyclin E were analyzed. The results following CTSD knockdown demonstrated significantly lower expression of the conversion factor cyclin A1 from the S phase to the G2/M phase. In contrast, the expression levels of a crucial cell cycle protein cyclin D2 in the G1 phase and the key conversion factor cyclin E in cells from the G0/G1 phase to the S phase were significantly and extremely high (P<0.01; Fig. 4h).


(a)
sh3-CTSD
sh-NC
CCK-8 OD450
Time (h)
(b)
PCNA mRNA relative expression ratio to β-actin
sh-NC
sh3-CTSD
(c)
sh-NC
A-0:P1
Q1-UL (0.17%)
Q1-UR (1.11%)
Q1-LL (96.26%)
Q1-LR (2.46%)
A-1:P1
Q1-UL (0.22%)
Q1-UR (1.26%)
Q1-LL (96.10%)
Q1-LR (2.42%)
A-2:P1
Q1-UL (0.33%)
Q1-UR (1.09%)
Q1-LL (96.47%)
Q1-LR (2.11%)
sh3-CTSD
A3-0:P1
Q1-UL (0.51%)
Q1-UR (0.70%)
Q1-LL (97.04%)
Q1-LR (1.74%)
A3-1:P1
Q1-UL (0.52%)
Q1-UR (0.73%)
Q1-LL (97.51%)
Q1-LR (1.24%)
A3-2:P1
Q1-UL (0.34%)
Q1-UR (0.73%)
Q1-LL (97.52%)
Q1-LR (1.41%)
PE-A
FITC-A
(d)
Apoptosis (%)
sh-NC
sh3-CTSD
(e)
mRNA relative expression ratio to β-actin
sh-NC
sh3-CTSD
Bcl-2
Bax
caspase-3

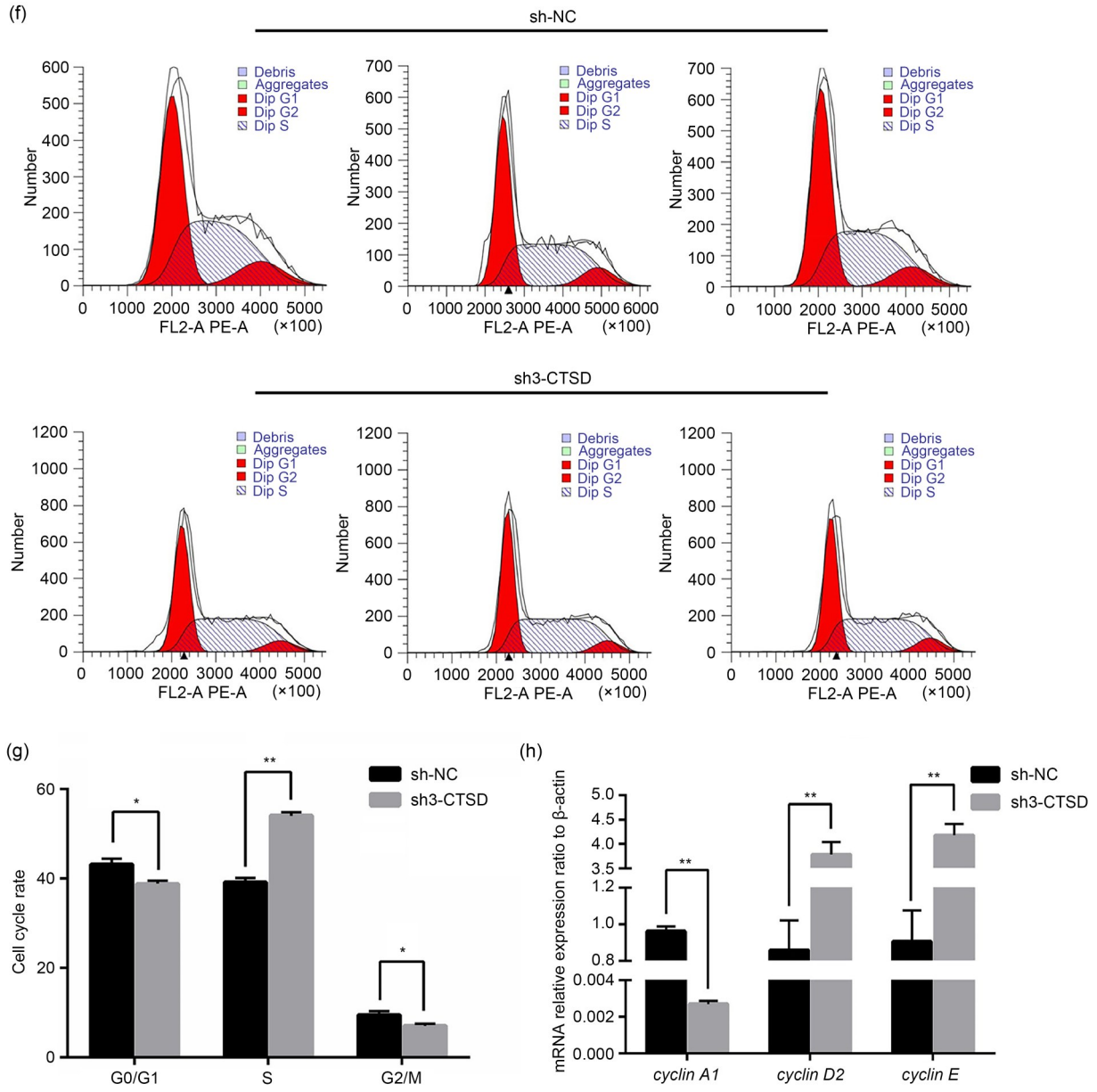


Fig. 4 Effects of cathepsin D (CTSD) knockdown on granulosa cell proliferation, apoptosis, cycle, and the expression of correlated genes. (a) Proliferative ability of granulosa cells based on the cell counting kit-8 (CCK-8) assay; (b) Effect of CTSD knockdown on the expression of proliferating cell nuclear antigen (*PCNA*) messenger RNA (mRNA) in granulosa cells; (c) Cell apoptosis as detected by flow cytometry; (d) Quantification of apoptotic cell number after transfection with sh3-CTSD or sh-NC; (e) Effect of CTSD knockdown on the expression of B cell lymphoma 2 (*Bcl-2*), Bcl-2-associated X (*Bax*), and *caspase-3* mRNAs in granulosa cells; (f) Cell cycle as detected by flow cytometry; (g) Quantification of cycle cell number after transfection with sh3-CTSD or sh-NC; (h) Effect of CTSD knockdown on the expression of *cyclin A1*, *cyclin D2*, and *cyclin E* mRNAs in granulosa cells. Data are presented as mean±standard deviation (SD), n=3. * P<0.05, ** P<0.01. sh-CTSD: CTSD short hairpin RNA (shRNA); sh-NC: negative shRNA control; OD_{450}: optical density at 450 nm; Dip: diploid.

4 Discussion

Notably, specificity was detected in the gene expression of different tissues and cells. Since the ovary contains a mixture of different cells, the expression of the candidate genes in these cells is not the same (Zhang et al., 2015). Morais et al. (2012) described the expression pattern of CTSD in the ovary, and

detected CTSD expression in the follicular cells at each stage of follicular growth and development. Moreover, the immunoreaction of CTSD was found in ovarian tissues of rats (Zhang et al., 2008). In the present study, we demonstrated that CTSD exists in the primordial, primary, and secondary follicles of "Qianbei Ma" goats and triggers positive immunoreaction in the corpus luteum and granulosa cells. This phenomenon verifies that CTSD is widely distributed in each part of goat ovary, whereas the positive expression of CTSD in each level of follicles demonstrates its potential contribution to the growth and development of goat follicle. Furthermore, the immunological competences of CTSD at all levels of follicle were distinct, whereas the immunocompetence in corpus luteum and big antral follicle before ovulation was higher. This result indicates that CTSD might be involved in follicular atresia in goats, as reported in other studies with different animal models (de Stasio et al., 1999; Leyria et al., 2018; Sales et al., 2019). Therefore, the detected expression pattern of CTSD in the goat ovary might offer novel perspectives for exploring the CTSD function and the regulatory mechanism of reproductive traits in goats.

This study demonstrated that CTSD expression in the ovaries of monotocous "Qianbei Ma" goats was significantly higher compared with the ovary of polytocous goats, as detected through western blotting. Similar results were reported by Feng et al. (2018). In recent years, the comparison of gene expression pattern between the ovaries of females with high or low reproductive capacity has aided in screening for genes related to litter size (Ling et al., 2014; Hu et al., 2020). The differential expression of CTSD gene in the ovarian tissues of monotocous and polytocous goats indicated that CTSD might have a high correlation with goat reproductive capacity, especially litter size. Previous research on the litter size trait has indicated that the differences in this trait are determined by hormone and genetic related genes (Yang et al., 2020). Numerous studies proved that the expression of *BMPR-IB*, *FSHR*, and *INHA* positively correlates with prolificacy in goats, and they may promote ovulation by regulating follicle development (Bradford, 1972; Mahdavi et al., 2014; Cui et al., 2019; Xia et al., 2020). In this study, we established that knocking down the expression of CTSD in granulosa cells significantly elevated the expression of *BMPR-IB*, *FSHR*, and *INHA* at both transcriptional and translational levels. Thus, we inferred that CTSD expression could inhibit the expression of BMPR-IB, FSHR, and INHA in granulosa cells, consequently impeding follicle growth and development, and in turn indirectly reducing the number of ovulations.

Follicular growth is characterized by the proliferation of granulosa cells and the enlargement of follicular volume. Meanwhile, follicle development is related to cell apoptosis, cell cycle, and the formation of vascular wall (Chou and Chen, 2018). CTSD is an extensively expressed aspartic protease associated with several biological processes, including cell proliferation and apoptosis (Tulone et al., 2007). The PCNA is a widely active cell proliferation marker in all cell types (including follicular granulosa cells), whose expression potentially improves cell proliferation (Strzalka and Ziemienowicz, 2011). Herein, the CCK-8 method was employed to reveal that CTSD knockdown in granulosa cells could enhance granulosa cell proliferation. Simultaneously, *PCNA* expression was significantly increased, indicating that CTSD expression could inhibit cell proliferation. Thus, we hypothesized that CTSD-induced cell proliferation might be associated with the expression level of *PCNA*.

Cell cycle regulation is extremely important for cell proliferation and differentiation, with cyclins being the critical regulatory factors for cell cycle progression (Chen et al., 2016). Through flow cytometry, we indicated that CTSD knockdown in granulosa cells significantly lowered the ratios of cells in the G0/G1 and G2/M phases, while it increased the ratio of cells in the S phase, indicating that CTSD knockdown could block granulosa cells in the S phase. We thus demonstrated that CTSD knockdown might improve granulosa cell proliferation, which was consistent with the CCK-8 detection results. Simultaneously, the expression levels of the important cell cycle regulatory factors *cyclin D2* and *cyclin E* were significantly elevated. On the other hand, the expression of *cyclin A1*, which has been shown to play a crucial role in cell cycle progression, was obviously decreased (Fuchimoto et al., 2001; Pradeep et al., 2002). Therefore, we speculated that CTSD potentially regulates the proliferation and differentiation of granulosa cells by varying the expression levels of *cyclin A1*, *cyclin D2*, and *cyclin E*, subsequently altering the progression and other functions of granulosa cells.

Cell apoptosis, a programmed cell death mechanism with distinctive characteristics, involves unique factors, such as members of the Bcl-2 protein family (including anti-apoptosis member Bcl-2 and pro-apoptosis member Bax). Notably, caspase-3 of the caspase factor family is the most important effector molecule in cell apoptosis, and is expressed in ovarian tissue and related cells (Greenfeld et al., 2007; Han et al., 2017). According to a previous research report, cell apoptosis is the primary mechanism for ovarian follicular atresia, and thus plays a crucial function in the ovarian follicle (Liu et al., 2018). This study established that the inhibition of CTSD activity could decrease granulosa cell apoptosis and elevate the expression of anti-apoptosis factor *Bcl-2*. In contrast, the expression of pro-apoptosis factors *Bax* and *caspase-3* was reduced. These findings indicated that, by changing the ratio between Bax and Bcl-2 in the Bcl-2 family, CTSD may cause mitochondrial dysfunction and further release the cell terminal apoptotic factor caspase-3, inducing the apoptosis of granulosa cells. Importantly, cell apoptosis induced by lysosomal cathepsin induces follicular atresia in mammals, whereas the increased CTSD activity potentially causes atresia in follicles (Gwon et al., 2017). The present study demonstrated that CTSD knockdown lowers granulosa cell apoptosis, and therefore, high CTSD expression is the essential reason for low productivity in goats.

5 Conclusions

This study demonstrated that CTSD knockdown potentially regulates granulosa cell proliferation, apoptosis, and cell cycle progression through a range of factors, including *PCNA*, *Bcl-2*, *Bax*, *caspase-3*, *cyclin A1*, *cyclin D2*, and *cyclin E*, which in turn affects the biological functions of granulosa cells. Besides, CTSD might affect follicle development and ovulation by regulating the candidate genes of the prolific trait, including *BMPR-IB*, *FSHR*, and *INHA*, thus indirectly influencing goat litter size.

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Author contributions

Zhinan ZHOU and Xiang CHEN conceived and designed the experimental program. Zhinan ZHOU, Min ZHU, Weiwei WANG, and Zheng AO wrote and edited the manuscript. Zhinan ZHOU, Wen TANG, and Lei HONG performed the experiments. Zhinan ZHOU and Jiafu ZHAO revised the manuscript. All authors have read and approved the final manuscript, and therefore, have full access to all data in this study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Zhinan ZHOU, Xiang CHEN, Min ZHU, Weiwei WANG, Zheng AO, Jiafu ZHAO, Wen TANG, and Lei HONG declare that they have no conflict of interest.

The animal handling procedures in this study adhered to the Animal Welfare Guidelines of the Animal Protection and Use Committee of Guizhou University, Guiyang, China (approval number: EGZU-2017T010). All animal handling procedures were performed to ensure minimal suffering.

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