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Research article

Overexpression of *CDC25C* affects the cell cycle of ovarian granulosa cells from adult and young goats

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ABSTRACT

Background: CDC25 is a dual-specificity phosphatase that was first identified in the yeast *Schizosaccharomyces pombe* as a cell cycle-defective mutant. Although CDC25 is involved in the cell cycle of ovarian granulosa cells, the CDC25 signaling pathway has not been clarified fully. To explore the role of *CDC25C* in the cell cycle of goat ovarian granulosa cells, a *CDC25C*-overexpressing vector, pCMV-HA-CDC25C, was constructed and transfected into granulosa cells from adult and young white goats from Jiangsu Nantong. RT-PCR was used to measure *CDC25C*, *CDK1*, and *WEE1* gene expression levels, and flow cytometry was used to distinguish ovarian granulosa cells in different phases of the cell cycle. Progesterone and estradiol levels in transfected ovarian granulosa cells were also measured.
Results: In adult goat follicular granulosa cells transfected with pCMV-HA-CDC25C, CDC25C expression increased significantly, which greatly increased the relative gene expression levels of both CDK1 and WEE1. Additionally, progesterone and estradiol levels were increased in goat follicular granulosa cells overexpressing CDC25C. And the cell cycle results showed that transfection of pCMV-HA-CDC25C leads to a higher proportion of cells in S phase compared to the no vector-transfected groups.
Conclusions: The results of this study indicated that the overexpression of *CDC25C* may increase the gene expression levels of both *WEE1* and *CDK1* in S phase and accelerate the transition of cells from G1 phase to S phase.

1. Introduction

Increasing the fecundity of livestock can increase the economic benefits of animal husbandry significantly, and *in vitro* fertilization is an effective method to improve such fecundity. *In vitro* fertilization typically uses oocytes from adult livestock, but oocytes from young livestock may both shorten the breeding interval and hasten the generation of genetic variants [1]. However, problems such as abnormal fertilizations and low blastocyst development rates occur with eggs from young livestock. Studies have indicated that these problems may be due to the immaturity or abnormal follicular development of oocytes from young livestock, leading to the abnormal development of oocytes or developmental retardation [2]. Therefore, more attention has recently been paid to the cell cycle and developmental differences between oocytes derived from lambs and adult goats.

Ovarian granulosa cells are located on the outside of the zona pellucida and are connected to the oocyte *via* gap junctions. Because numerous hormones and growth factors secreted by granulosa cells affect the growth and maturation of oocytes [3], it is important to investigate the cell cycle of ovarian granulosa cells to optimize livestock breeding. Previous studies have shown that numerous genes are involved in the cell cycle [4,5]. The cell division cycle 25 homolog C gene, *CDC25C*, encodes a conserved protein that plays a key role in the regulation of cell division [6,7,8]. CDC25C activates CDK2 and CDK1, which are essential for mitosis [9,10,11]. Single-cell transcriptome sequence technology showed that the expression of *CDC25C* in adult ewes is eight times higher than in young ewes [15]. *WEE1* encodes a nuclear serine/threonine kinase from the fission yeast *Schizosaccharomyces pombe* that has been shown to inhibit CDK1 and entry into mitosis, and affect cell size. WEE1 also regulates DNA replication, histone transcription, and chromosome condensation [12, 13], and is very active in the S and G2 phases of the cell cycle [14]. The cell cycle is a complex biological process in which any abnormal gene expression is likely to lead to unregulated cell growth, blockade of differentiation, abnormal apoptosis, *etc.* The regulation of the *CDC25C*, *CDK1*, and *WEE1* genes, key mediators of the cell cycle, has been the

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primary focus of current cell cycle research. To explore the cell cycle of maturing oocytes and the role of *CDC25C* in ovarian development, we overexpressed *CDC25C* in ovarian granulosa cells using a recombinant plasmid expressing *CDC25C*, pCMV-HA-CDC25C.

2. Materials and methods

2.1. Test animals and sample collection

The animal experiments were approved by the Yangzhou University Ethics Committee, and the animals used were in accordance with the national standards of China. The goats were obtained from a goat market in Hai'an County, Nantong City, Jiangsu Province. Ovaries were collected from several healthy native Jiangsu white goats, 1–2-year-old adult ewes, and 1–2-month-old lambs, and freshly obtained ovarian tissues were immersed in precooled 10 mM phosphate-buffered saline (PBS) and returned to the laboratory within 1–2 h.

2.2. Construction of the recombinant overexpression vector

2.2.1. RNA extraction and reverse transcription

Total RNA was extracted from ovaries using the Trizol reagent; RNA concentration and purity were determined by UV spectrophotometry; and RNA was stored at -80°C. cDNA was synthesized by reverse transcription using the FastQuant RT kit [Tiangen Biotech (Beijing) Co., Ltd.].

2.2.2. Primer design and synthesis

Primers for the coding region of *CDC25C* were designed based on the *CDC25C* goat sequence (NCBI GeneID: 102187214), using Primer5-blast. The primer sequences used were F:5′-AGTCGACCATGTCTGCAGAATTCT CATC-3′ and R:5′-GCGGCCGCTCATGGGCTCACATCCTTCAC-3′, and the primers were synthesized by Shanghai Sangon Biological Co., Ltd. The PCR product length was 1170 bp.

2.2.3. PCR amplification and recovery of target strips

PCR amplification was performed using cDNA as a template. The amplification reaction consisted of 1 μg of the cDNA template, 1 μL of the forward primer, 1 μL of the reverse primer, 1 μL of 2× *Taq* PCR MasterMix, and RNase-free ddH_2O, in a total volume of 20 μL. Amplification was performed as follows: 3 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 58°C, and 40 s at 72°C; and a 5 min extension at 72°C. PCR products were detected by 1% agarose gel electrophoresis, recovered, and purified using the TIANgel Midi Purification kit (Tiangen Biotech).

2.2.4. Construction of pCMV-HA-CDC25C

The resultant *CDC25C* gene fragment was ligated into a pCMV-HA vector that had been doubly digested with *Sal*1 and *Not*l, and the ligation was transformed into *Escherichia coli* DH5α competent cells. The recombinant plasmid was extracted from a transformant using the HighPure Maxi Plasmid kit (Tiangen Biotech), and the insertion was confirmed by PCR. Plasmids containing the insert were sent for sequencing (Shanghai Sangon Biological Co., Ltd.), and the sequences were analyzed using DNAstar software.

2.3. Isolation and culture of goat ovary granulosa cells

Goat granulosa cells were isolated and cultured according to Caloni et al. [16] and Zhu et al. [17] using a disposable syringe to pierce and absorb the follicular fluid and avoid blood vessels. Cells were washed three times with PBS, resuspended in DMEM/F12 media, stained with trypan blue, and counted. After adjusting for cell density, cells were cultured at 37°C, under humidification and 5% CO_2.

2.4. Granular cell staining and immunofluorescent staining

Granulocytes were stained with neutral red, observed, and photographed. Granulosa cells were also stained, using an immunohistochemistry kit (Boster Biological Technology Co., Ltd.), according to the manufacturer's instructions. The primary antibody used was rabbit anti-follicle-stimulating hormone receptor (FSHR) polyclonal antibody, and the secondary antibody used was SA1074 anti-rabbit IgG. Immunohistochemically stained cells were observed using inverted fluorescence microscopy. The FSHR protein appeared red.

2.5. Cell transfection

Transfection was performed by the addition of vector and the FuGENE® HD transfection reagent to goat ovary granulosa cells after 24 h of culture when the cell density reached approximately 60%, according to the manufacturer's instructions (Promega). The pCMV-HA-CDC25C:transfection reagent (FuGENE) ratio was 3:1.

2.6. Quantitative fluorescence PCR

The relative gene expression levels of *CDC25C*, *CDK1*, and *WEE1* were assessed in six different groups: the no vector-transfected adult goat group, the empty vector-transfected adult goat group, the pCMV-HA-CDC25C-transfected adult goat group, the no vector-transfected young goat group, the empty vector-transfected young goat group, and the pCMV-HA-CDC25C-transfected young goat group, by quantitative fluorescence PCR, using the primers shown in Table 1. The expression level of each gene in each group was measured three times, the data were analyzed using ABI 7500 quantitative fluorescence PCR software, v2.0.6 (Applied Biosystems), and the $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression levels.

2.7. Progesterone and estradiol levels

Progesterone and estradiol levels were measured by ELISA [YanSheng Biotech (Shanghai) Co., Ltd.], according to the manufacturer's instructions.

2.8. Cell cycle and apoptosis analysis

Cell cycle and apoptosis analysis kits (Beyotime Biotechnology Co., Ltd.) were used to analyze the cell cycle and apoptosis of ovarian granulosa cells from both adult and young ewes. Flow cytometry was used to distinguish cells in different phases of the cell cycle.

2.9. Statistical analysis

The Student's *t* test (parametric and unpaired) was used to determine the significance of the results with SPSS software, version 18.0 (SPSS, Inc., Chicago, IL, USA). All data are presented as the

Table 1
Primer sequences used in fluorescence quantitative PCR.

Gene	Primer sequence (5′-3′)	Product size (bp)	Annealing temperature (°C)
CDC25C	F: TGGAGTCTACAGGACCTGAGCAA R: TGGGACTGCCCAGATGTTTCA	215	58
CDK1	F: TATTGCTGGGGTCAGCTCGC R: TCCACTTCTGGCCACACTTCA	180	58
WEE1	F: CATTCTGCTCATCAACAGAGCCAG R: ACATGGCATCTCACGATATTTGG	239	59
β-Actin	F: AGCAGTCGTTGGAGCGAG R: ACATGGCATCTCACGATATTTGG	125	58

mean ± SD, and *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Construction of pCMV-HA-CDC25C

The coding regions of *CDC25C* were amplified by PCR, using cDNA as a template, and the PCR products were analyzed by agarose gel electrophoresis (Fig. 1). A 1170 bp PCR product was observed, as expected. pCMV-HA-CDC25C plasmid DNA was extracted from bacterial transformants and digested with restriction enzymes; the presence of two fragments (1170 bp and 3.8 kb) confirmed that the *CDC25C* insertion was present and that the recombinant vector has been constructed successfully (Fig. 2). Sequencing analysis confirmed that the coding sequence of *CDC25C* in pCMV-HA-CDC25C matched the coding sequence of *CDC25C* in the NCBI database. The pCMV-HA vector plasmid map is shown in Fig. 3.

3.2. Quantitative fluorescence PCR

There was no difference ($P \geq 0.05$) in the relative expression of *CDC25C* in granulosa cells from the no vector-transfection adult goat group, the empty vector-transfection adult goat group, the no vector-transfection young goat group, and the empty vector-transfection young goat group. The relative expression of *CDC25C* in granulosa cells increased 160-fold in the pCMV-HA-CDC25C-transfected adult goat group compared to the no vector-transfection adult goat group ($P < 0.05$). The relative expression of *CDC25C* in granulosa cells increased 21-fold in the pCMV-HA-CDC25C-transfected young goat group compared to the no vector-transfection young goat group ($P < 0.05$) (Fig. 4a). These results indicate that pCMV-HA-CDC25C was successfully transfected and expressed in goat follicular granulosa cells.

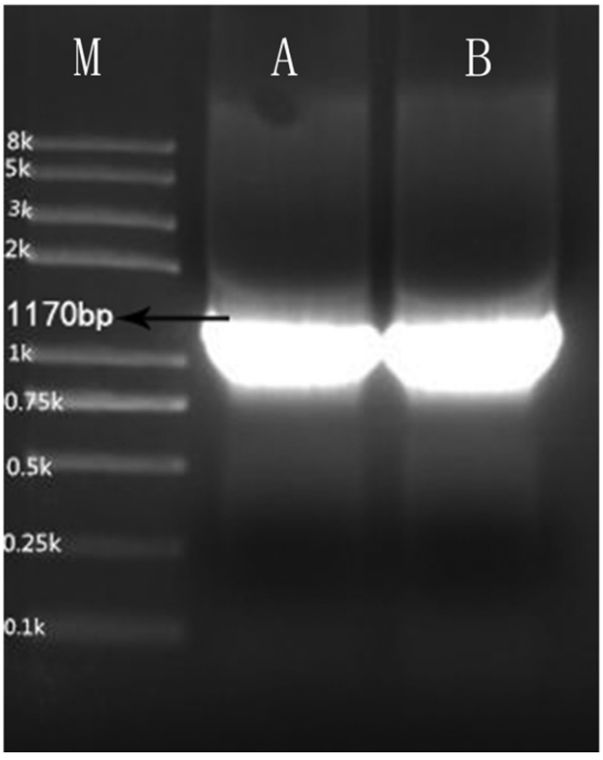

Fig. 1. PCR amplification of *CDC25C*. Lane M shows the molecular weight markers; lanes A and B show the *CDC25C* gene.

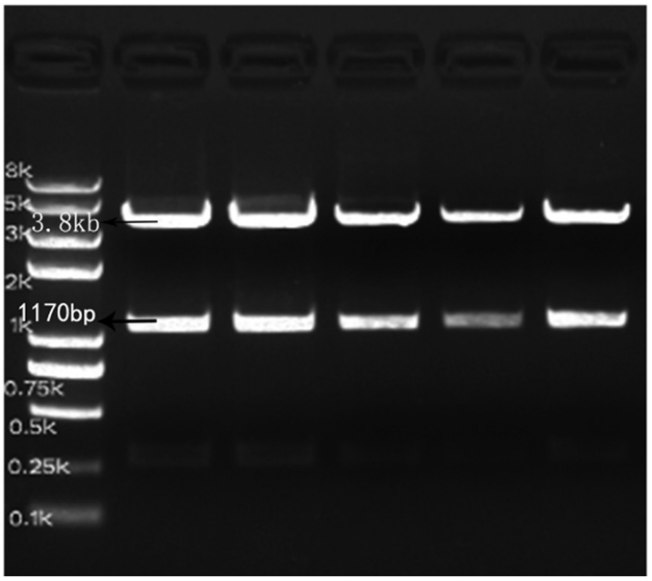

Fig. 2. Restriction digest of pCMV-HA-CDC25C isolated from bacteria. After the pCMV-HA-CDC25C plasmid was digested with restriction enzymes, two fragments (1170 bp and 3.8 kb) were obtained.

In adult goat follicular granulosa cells transfected with pCMV-HA-CDC25C, *CDK1* expression also increased, presumably as a result of *CDC25C* overexpression ($P < 0.05$; Fig. 4b). In contrast, in young goat follicular granulosa cells, *CDK1* expression was lower in the empty vector-transfection and pCMV-HA-CDC25C-transfection groups than in the no vector-transfection group ($P < 0.05$).

In adult goat follicular granulose cells, *WEE1* expression was lower in the empty vector-transfection group than in the no vector-transfection group ($P < 0.05$; Fig. 4c), whereas *WEE1* expression was higher

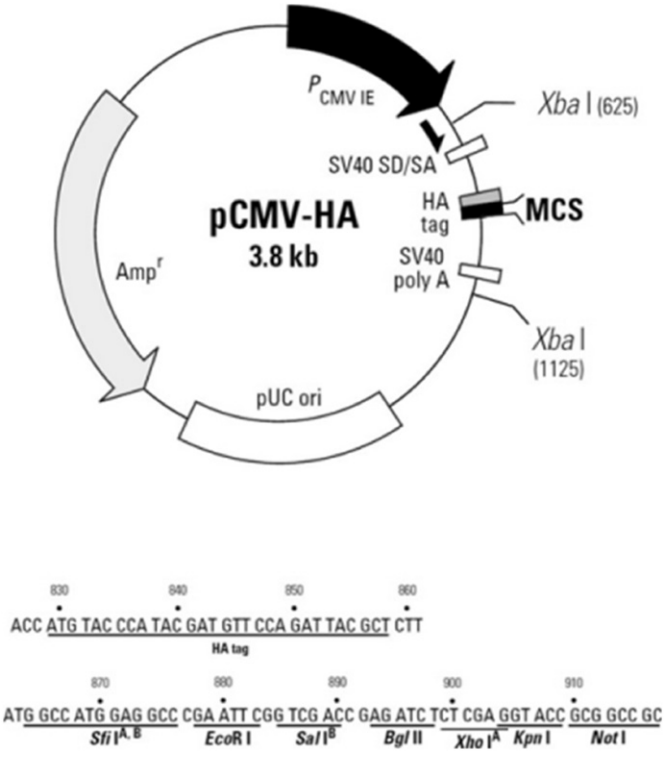

Fig. 3. pCMV-HA plasmid map and sequence of the multiple cloning site (MCS).

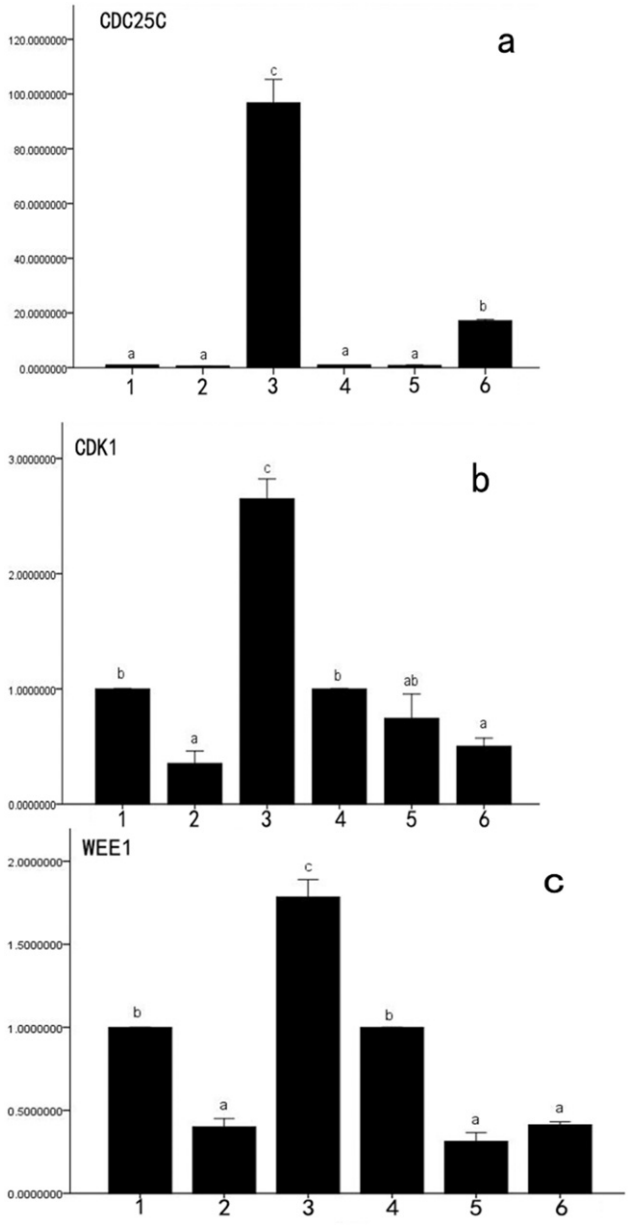

Fig. 4. Relative gene expression levels by quantitative fluorescence PCR. Relative expression levels of *CDC25C* (a), *CDK1* (b), and *WEE1* (c) are shown. 1: No vector-transfection adult goat group, 2: empty vector-transfection adult goat group, 3: pCMV-HA-CDC25C-transfected adult goat group, 4: no vector-transfection young goat group, 5: empty vector-transfection young goat group, and 6: pCMV-HA-CDC25C-transfected young goat group. Different letters above the bars indicates a significant difference ($P < 0.05$), whereas the same letters above the bars indicates no significant difference ($P \geq 0.05$).

in the pCMV-HA-CDC25C-transfection group than in the empty vector-transfection and no vector-transfection groups ($P < 0.05$; Fig. 4c). In young goat follicular granulosa cells, *WEE1* expression was lower in the pCMV-HA-CDC25C-transfection and empty vector-transfection groups than in the no vector-transfection group ($P < 0.05$; Fig. 4c).

3.3. Goat granulosa cell staining in vitro

After staining and visualization by light microscopy, the peripheries of goat granulosa cells appeared red, and the cells, which appeared normal and healthy (Fig. 5), were used in subsequent transfection experiments.

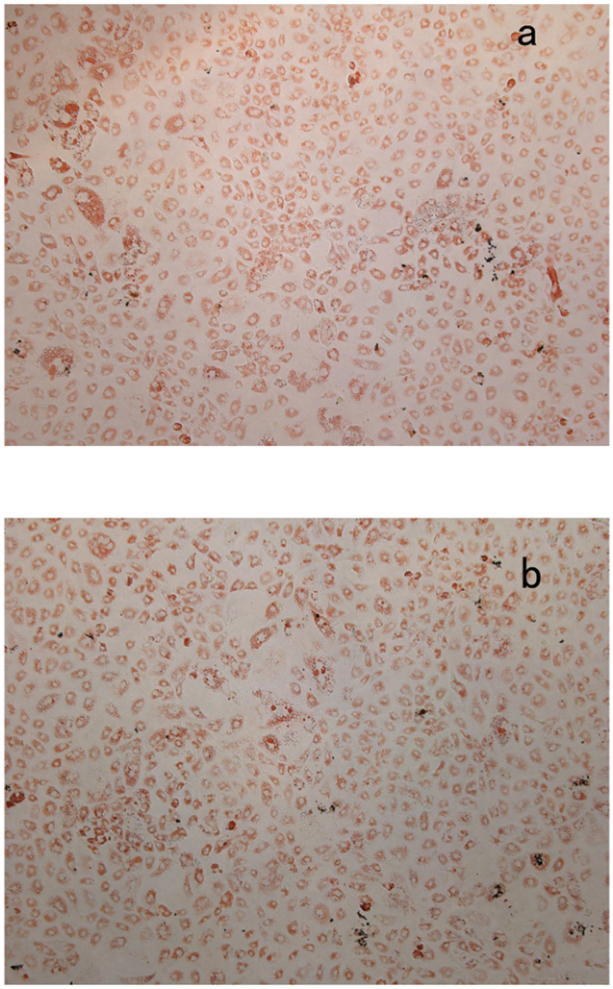

Fig. 5. Neutral red staining of ovarian granulosa cells (400×). (a) Ovarian granulosa cells from adult ewes. (b) Ovarian granulosa cells from young ewes.

3.4. Immunofluorescence

Immunofluorescence results confirmed that cultured goat follicular granulosa cells expressed FSHR (Fig. 6). This finding indicates that goat ovarian granulosa cells were successfully cultured.

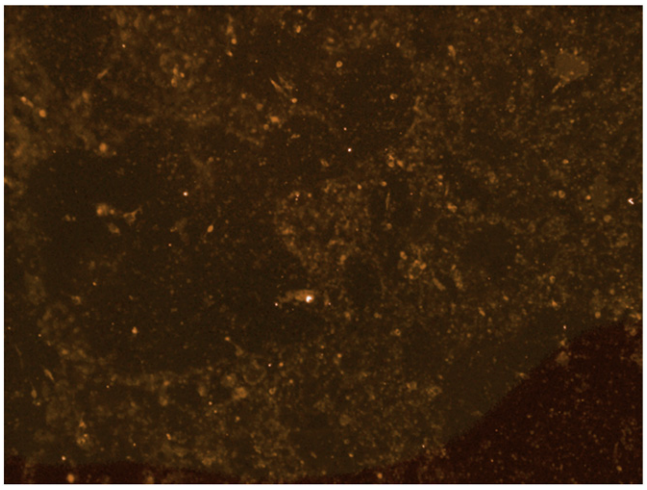

Fig. 6. Immunofluorescence of ovarian granulosa cells (100×). Cellular immunofluorescence of cells observed using inverted fluorescence microscopy. The FSHR protein appears red.

3.5. Progesterone and estradiol levels in goat ovarian cells

Progesterone and estradiol play important roles in the overall development of ovarian follicles. Estrogen is mainly derived from theca and granulosa cells, whereas progesterone is mainly produced by the corpus luteum within the follicles and formed by follicular granulosa cells. We found that progesterone and estradiol levels were higher in ovarian cells from the pCMV-HA-CDC25C-transfected adult and young goat groups than from the no vector-transfection adult and young goat groups (Fig. 7a and b). The progesterone level was higher in ovarian cells from the pCMV-HA-CDC25C-transfected adult goat group than from the pCMV-HA-CDC25C-transfected young goat group; we speculate that this may be because adult goat follicular granulosa cells are more likely to undergo luteinization *in vitro*.

3.6. Cell cycle

In the pCMV-HA-CDC25C-transfected adult goat group, the proportion of cells in G1 phase was 91.44%, the percentage of cells in S phase was 5.68%, and the proportion of cells in G2 phase was 2.89% (Fig. 8a). In the pCMV-HA-CDC25C-transfected young goat group, the percentages of cells in the G1, S, and G2 phases were 93.98%, 4.71%, and 1.31%, respectively (Fig. 8b). In the no vector-transfected adult goat group, the proportion of cells in G1 phase was 94.27%, with 1.66% and 4.08% of cells in the S and G2 phases, respectively (Fig. 8c). In the no vector-transfected young goat group, the proportion of cells in the G1, S, and G2 phases was 95.35%, 1.55%, and 3.10%, respectively (Fig. 8d). From these results, we know that transfection of pCMV-HA-CDC25C leads to a higher proportion of cells in S phase compared to the no vector-transfected groups.

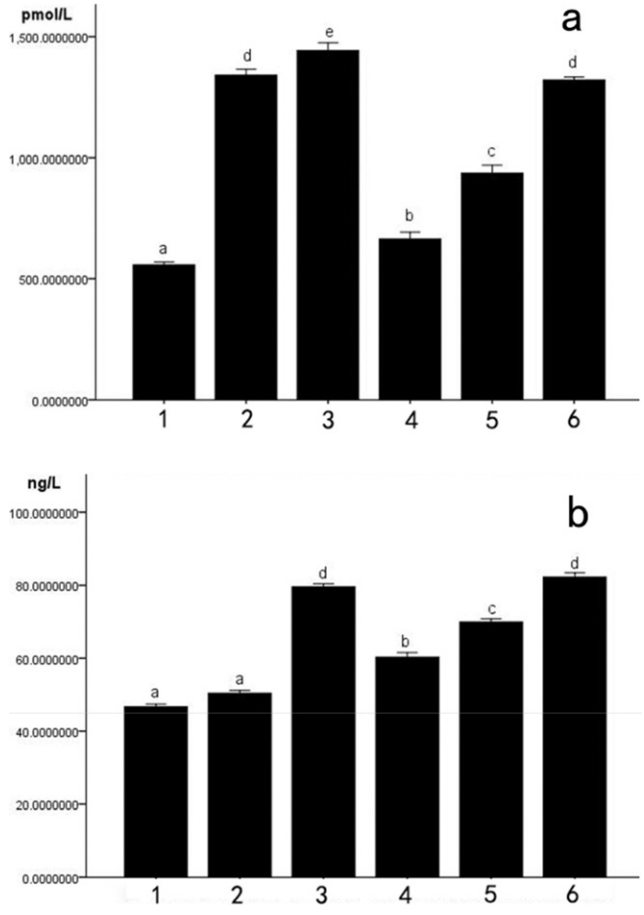

Fig. 7. Progesterone and estradiol levels. Progesterone (a) and estradiol (b) levels are shown. 1: No vector-transfection adult goat group, 2: empty vector-transfection adult goat group, 3: pCMV-HA-CDC25C-transfected adult goat group, 4: no vector-transfection young goat group, 5: empty vector-transfection young goat group, and 6: pCMV-HA-CDC25C-transfected young goat group. Different letters above the bars indicate a significant difference ($P < 0.05$), whereas the same letters above the bars indicate no significant difference ($P \geq 0.05$).

4. Discussion

To explore the role of *CDC25C* in goat follicular granulosa cells, we overexpressed the *CDC25C* gene and assessed not only its expression but also that of *CDK1* and *WEE1*. The results of this study show that in adult goat follicular granulosa cells transfected with pCMV-HA-CDC25C, *CDC25C* expression increased significantly, which greatly increased the relative gene expression levels of both *CDK1* and *WEE1*. Additionally, we found that progesterone and estradiol levels were increased in goat follicular granulosa cells overexpressing *CDC25C*.

The phases of the cell cycle include G1, S, G2, and M. Cells carry out the necessary preparations for growth during the G1 phase. S phase is the period during which DNA is synthesized, whereas the G2/M checkpoint is when the accuracy of the genetic material after replication is ensured. M phase is when cells divide their genetic material for the next generation of cells and then complete cell division [18]. The cell cycle is a complex process, which is controlled by cyclins, CDKIs, and CDKs. CDKs combine with cyclins to promote cellular proliferation [19].

The *CDC25C* gene is a key mediator of the cell cycle and plays an important regulatory role at the G2/M checkpoint [20,21]. During the beginning of M phase, CDC25C dephosphorylates Cdc2 at Y14/Y15, thereby activating the Cdc2/cyclin B1 complex to initiate mitosis [22]. Studies have found that when CDC25C phosphatase activity is inhibited, the activity of the Cdc2/cyclin B complex is also inhibited, resulting in G2/M phase arrest [23,24]. The expression level of CDC25C in cancer tissues is significantly higher than in normal tissues [25], suggesting that aberrant expression of CDC25C may induce cellular carcinogenesis and that high expression levels of CDC25C may accelerate cancer progression. In this study, we found that the overexpression of *CDC25C* increased the proportion of cells in S phase and accelerated the transition of cells from G1 phase to S phase.

The *CDK1* gene is a key regulator of the cell cycle, plays important roles in both M phase and the G2/M checkpoint, and participates in G1 phase cellular activity. During M phase, cyclins and CDKs form cyclin-CDK complexes. The activation of these complexes by phosphorylation by WEE1 regulates the process of mitosis [26,27]. The results of this study showed that the upregulation of CDC25C increases the expression of WEE1, likely because of the increase in the proportion of cells in S phase during which the expression of the *WEE1* gene also increases. WEE1 plays an important regulatory role during S phase [28]. In G1/S phase, CDKs initiate DNA replication; in S phase, WEE1 phosphorylates CDK1 and H2B, regulating histone synthesis and entry into mitosis [29]. These results suggest that *CDK1* expression levels increase in S phase, which is consistent with the results of this study, in which the upregulation of *CDC25C* increases both the proportion of cells in S phase and the expression levels of *CDK1*.

CDC25C, CDK1, and WEE1 interact with each other to regulate the cell cycle. CDK1 forms a complex with the regulatory subunit B1 to promote mitosis. However, during interphase, WEE1 phosphorylates Thr14 and Tyr15 of CDK1, thereby inactivating the B1-CDK1 complex [30]. Dephosphorylation of CDK1 on Tyr15 and Thr14 by CDC25C is important for CDK1 activation and G2/M phase conversion. WEE1 delays mitosis by phosphorylating CDK1, whereas CDC25C promotes mitosis by dephosphorylating CDK1 [31]. It has been shown that CDC25-related phosphatases reverse the inhibitory phosphorylation of CDKs by WEE1-related kinases, thereby promoting entry into mitosis.

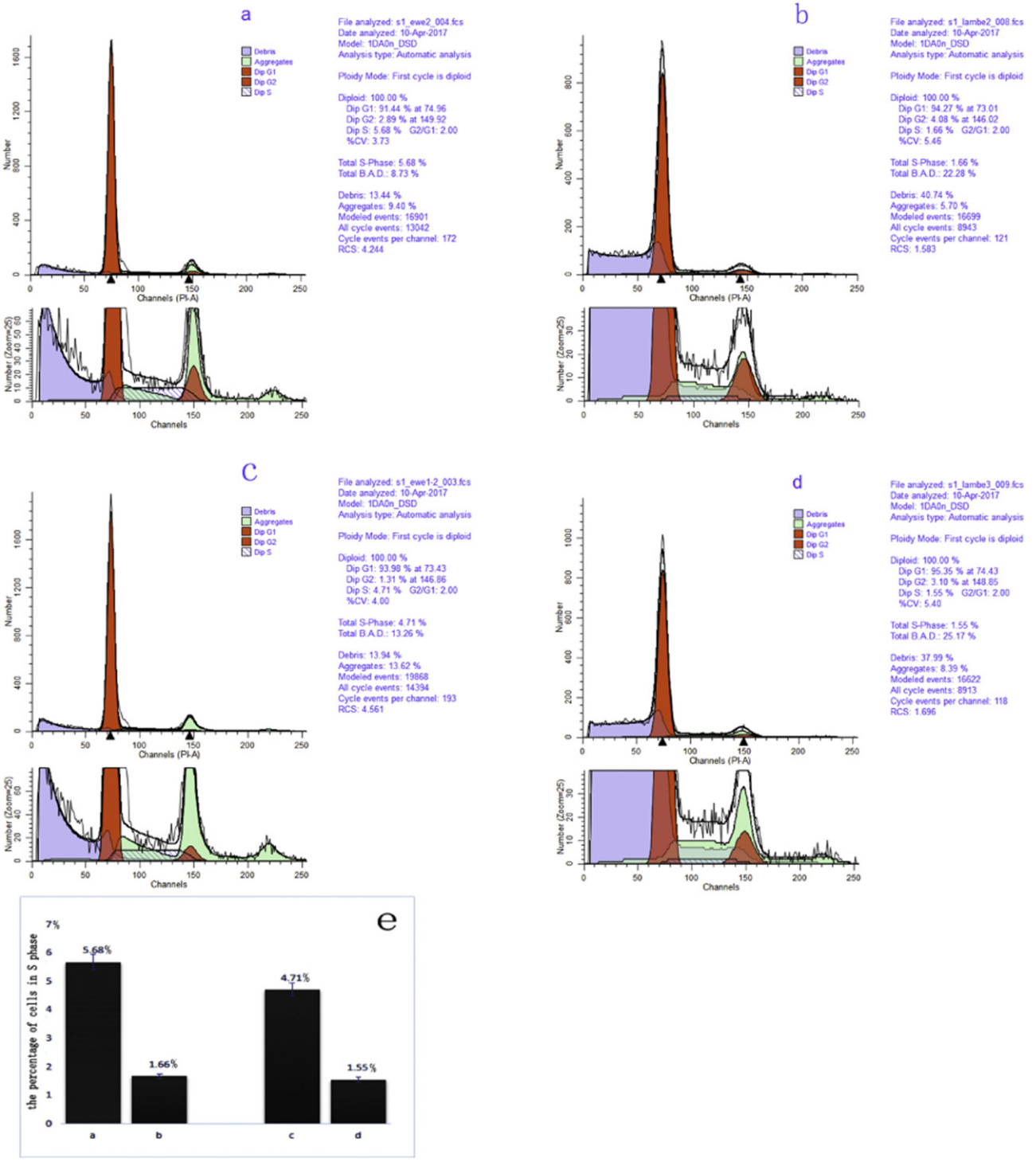

Fig. 8. Cell cycle analysis. (a) pCMV-HA-CDC25C-transfected adult goat group, (b) no vector-transfection adult goat group, (c) pCMV-HA-CDC25C-transfected young goat group, (d) no vector-transfection young goat group, and (e) the percentage of cells in S phase.

In addition, the overexpression of WEE1 and inhibition of CDC25C increases the phosphorylation of CDK1 on Tyr15, leading to G2 arrest [32].

In the present study, the upregulation of *CDC25C* significantly increases the relative gene expression levels of both *CDK1* and *WEE1*. In addition, the transfection of pCMV-HA-CDC25C into follicular granulosa cells increases the proportion of cells in S phase and lowers the percentage of cells in G1 phase. These results indicate that the overexpression of *CDC25C* may accelerate the transition of cells from G1 phase to S phase.

Conflict of interest

The authors declare no conflict of interest.

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