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Roles of vitamin D and its receptor in the proliferation and apoptosis of luteinised granulosa cells in the goat

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Abstract. The objective of this study was to investigate the dose-dependent effect of 1α,25-$(OH)_2VD_3$ (Vit D_3) on *in vitro* proliferation of goat luteinised granulosa cells (LGCs) and to determine the underlying mechanisms of its action by overexpressing and silencing vitamin D receptor (VDR) in LGCs. Results showed that VDR was prominently localised in GCs and theca cells (TCs) and its expression increased with follicle diameter, but was lower in atretic follicles than in healthy follicles. The proliferation rate of LGCs was significantly higher in the Vit D_3-treated groups than in the control group, with the highest proliferation rate observed in the 10 nM group; this was accompanied by changes in the expression of cell cycle-related genes. These data indicate that Vit D_3 affects LGC proliferation in a dose-dependent manner. Contrary to the VDR knockdown effects, its overexpression upregulated and downregulated cell cycle- and apoptosis-related genes respectively; moreover, supplementation with 10 nM of Vit D_3 significantly enhanced these effects. These results suggest that changes in VDR expression patterns in LGCs may be associated with follicular development by regulation of cell proliferation and apoptosis. These findings will enhance the understanding of the roles of Vit D_3 and VDR in goat ovarian follicular development.

Additional keywords: 1α,25-$(OH)_2VD_3$, follicular development, ovary.

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Introduction

The active form of vitamin D, 1α,25-$(OH)_2VD_3$ (vitamin D_3, henceforth referred to as Vit D_3), is a member of the family of steroid hormones and is known to perform a wide spectrum of biological functions (Suda 2010). All the genome-level actions of Vit D_3 are mediated by the vitamin D receptor (VDR), which is a member of the nuclear steroid hormone receptor superfamily (Carlberg and Campbell 2013). Consequently, Vit D_3 deficiency is associated with various disorders of female reproduction such as reduced fertility, arrested follicular development, ovulatory dysfunction and delayed oestrous cycle (Lorenzen *et al.* 2017; Pilz *et al.* 2018). The vital roles of Vit D_3 have been demonstrated by studying VDR in human female reproductive organs such as the placenta (Pospechova *et al.* 2009; Nguyen *et al.* 2015), uterus (Viganò *et al.* 2006) and ovaries (Parikh *et al.* 2010). Likewise, the roles of VDR were evidenced by its detection in animal studies such as that in the placenta of mice (Shahbazi *et al.* 2011), uterus of mice (Zarnani *et al.* 2010), pigs (Grzesiak *et al.* 2019) and buffaloes (Emam *et al.* 2016) and ovaries of mice (Shahbazi *et al.* 2011) and goats (Yao *et al.* 2017*b*). These findings suggest that Vit D_3 plays an important role in female reproduction.

The localisation of VDR in ovarian granulosa cells (GCs; Shahbazi *et al.* 2011; Wojtusik and Johnson 2012; Herian *et al.* 2018) suggests its importance in the process of folliculogenesis, which was confirmed in VDR-null female mice that showed uterine hypoplasia along with impaired folliculogenesis and complete infertility despite dietary Vit D_3 supplementation (Yoshizawa *et al.* 1997; Kinuta *et al.* 2000). Nevertheless, VDR could also perform some functions independent of its ligand (Vit D_3); for example, it is known to be involved in calcium metabolism, cell proliferation and apoptosis (Carlberg and Campbell 2013; Oda *et al.* 2018). Therefore, we hypothesised that VDR plays an important role in follicular development by regulating GC proliferation and apoptosis, either dependently or independently of its ligand. In mammals, only a few follicles ovulate and more than 99% of follicles do not mature fully, but instead undergo atresia during the developmental process (Asselin *et al.* 2000; Matsuda *et al.* 2012). The excessive rate of follicular atresia and apoptosis restricts the number of

Table 1. Information about the antibodies used in the study
IHC, immunohistochemistry; IF, immunofluorescence; WB, western blotting

Antibody	Cat no.	Company	Dilution for IHC/IF	Dilution for WB
VDR	14526–1-AP	ProteinTech, Chicago, USA	1 : 200	1 : 500
Bcl-2	12789–1-AP	ProteinTech, Chicago, USA	–	1 : 1000
BAX	50599–2-lg	ProteinTech, Chicago, USA	–	1 : 2000
p27	25614–1-AP	ProteinTech, Chicago, USA	–	1 : 1000
β-actin	bs-0061R	Bioss, Beijing, China	–	1 : 2000
Goat anti-rabbit lgG	A0208	Beyotime Biotechnology, Nantong, China	1 : 100	1 : 1000
Goat anti-rabbit Alexa Fluor 594	B40925	Invitrogen, Carlsbad, CA, USA	1 : 200	–

developed and ovulated follicles, which may significantly impair the stimulatory actions of follicular steroids on female reproduction (Kaipia and Hsueh 1997). Thus, elucidating the local mechanism of VDR action in follicular development may explain the effects of Vit D_3 on female fertility, especially on the proliferation and apoptosis of GCs.

Increasing evidence indicates that Vit D_3 modulates follicular development (Xu *et al.* 2018), which may occur via regulation of cell cycle- and apoptosis-related genes (Samuel and Sitrin 2008; Irani and Merhi 2014); Vit D_3 may also act on trophoblast cells through placental VDR (Knabl *et al.* 2017; Nguyen *et al.* 2018). Our previous study was the first to suggest that an appropriate amount of Vit D_3 can promote proliferation of luteinised granulosa cells (LGCs) by regulating cell cycle- and antioxidant-related genes (Yao *et al.* 2017*b*). Nevertheless, dose-dependent responses of goat LGCs to Vit D_3 still need to be explored.

Therefore, in this study, we used goat LGCs as an experimental model to test whether Vit D_3 has a dose-dependent influence on the proliferation of LGCs. Furthermore, we determined VDR expression patterns in follicles of different sizes, either healthy or atretic, and identified its underlying mechanism by overexpressing or silencing VDR in LGCs. This study will not only improve our understanding of the roles of Vit D_3 and VDR in follicular development but also suggests effective amounts of Vit D_3 that could influence follicular development as reference for calculating concentrations of dietary Vit D_3 supplements for non-pregnant goats.

Materials and methods

Reagents and ethics

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich and culture media were procured from Life Technologies. All antibodies were obtained from commercial sources (Table 1). The study protocol was approved by the Institutional Animal Care and Use Committee of the Nanjing Agricultural University (SYXK2011–0036).

Animals and sample collection

The Yangtze River Delta white goat (non-pregnant) was used as a model for our study. During the breeding season (October to March of consecutive years), goat ovaries were collected from a local abattoir (Taizhou, Jiangsu, China; 32°00′N, 119°57′E), immediately immersed in sterile physiological saline (at 30–35°C) supplemented with 100 IU mL^{-1} penicillin and 50 μg mL^{-1} streptomycin and transported to the laboratory within 2 h. The connective tissues and attached oviducts were removed after five washes with Dulbecco's phosphate-buffered saline (DPBS). For immunohistochemical assay, five ovaries were randomly selected, fixed in 4% formaldehyde for 24 h and then embedded in paraffin. For VDR mRNA and protein analysis in follicles of different sizes, all visible antral follicles from ~20 ovaries were dissected, measured with a caliper and classified into three sizes (≤2, 2–5 and ≥5 mm). The follicle state (healthy or atretic) was determined based on morphological criteria using a surgical dissecting microscope as previously described (Moor *et al.* 1978). In total, 20 large follicles (≥5 mm; 12 healthy and 8 atretic), 40 medium follicles (2–5 mm; 18 healthy and 22 atretic) and 68 small follicles (≤2 mm; 40 healthy and 28 atretic) were collected. Subsequently, for each analysis (for different sizes of healthy or atretic follicles) of VDR mRNA and protein, three follicles were randomly selected from large follicles and three pooled samples were prepared from all collected categories of the medium and small follicles. It is well known that the GCs of various sizes of follicles have different shapes and transcriptome profiles at different follicular development stages (Hatzirodos *et al.* 2014). Therefore, GCs were further isolated only from medium follicles (2–5 mm) for the *in vitro* experiments.

Isolation of goat GCs

Goat GCs were harvested from the 2–5 mm healthy follicles using our previously described method (Zhang *et al.* 2016). Briefly, GCs were aspirated with a micropipette, transferred to 15 mL centrifuge tubes and centrifuged at 1500*g* for 5 min at room temperature (about 25°C). Next, the supernatant (follicular fluid) was discarded and GC pellets were resuspended in red blood cell lysis buffer for 1 min. After washing with DPBS, the cell pellets were resuspended in 0.3% hyaluronidase for 90 s. Finally, the GCs were resuspended in basic culture medium (BCM; Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum (FBS), 100 IU mL^{-1} penicillin and 100 g mL^{-1} streptomycin). The isolated GCs were added to T25 culture flasks at 5×10^4 cells cm^{-2} and incubated at 37°C and 5% CO_2. After 48 h of cultivation with serum, most cells converted into LGCs (Tosca *et al.* 2010).

Table 2. Sequences of the three siRNA used in this study
S: sense; A, anti-sense

Name	Sequence (5′–3′)
NC	S:5′-UUCUCCGAACGUGUCACGUTT-3′
	A:5′-ACGUGACACGUUCGGAGAATT-3′
siRNA1 (VDR-393)	S:5′-GCUUCCAUUUCAACGCUAUTT-3′
	A:5′-AUAGCGUUGAAAUGGAAGCTT-3′
siRNA2 (VDR-1075)	S:5′-GCUGAAGUCGAGUGCCAUUTT-3′
	A:5′-AAUGGCACUCGACUUCAGCTT-3′
siRNA3 (VDR-1159)	S:5′-CCAGGACUACAAGUACCAATT-3′
	A:5′-UUGGUACUUGUAGUCCUGGTT-3′

Experimental design of in vitro *LGC culture*

The first experiment was conducted to test whether Vit D_3 has a dose-dependent effect on the *in vitro* proliferation of goat LGCs. A total of 5×10^5 cells per well were seeded in 6-well plates in BCM and incubated for 24 h. The medium was replaced with fresh BCM containing various concentrations of Vit D_3 (0, 1, 10 or 100 nM; Selleck) and the cells were further incubated at 37°C and 5% CO_2 for 48 h. LGCs were concentrated by centrifuging at 5000*g* for 5 min at - room temperature (about 25°C) to remove the culture medium before storing the cells at −80°C for further analyses.

The second experiment was designed to investigate the mechanism of action of VDR by overexpression or silencing VDR in LGCs. The overexpression vector (derived from the pEX-4 vector) and small interfering RNA (siRNA) of VDR were synthesised by GenePharma; the siRNA targeted the intron–exon junction region of the VDR sequence. For overexpression, the full-length mRNA sequence of the VDR gene (GenBank accession number: KY307887.1) was used. For silencing, three siRNAs were synthesised (the sequences are listed in Table 2). The pEX-4-VDR plasmid or siRNAs were transfected into goat LGCs using Lipofectamine 2000 (Life Technologies). Next, 48 h after the transfection, the cells were collected to confirm the transfection efficiency by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and western blotting. The transfected LGCs were then subjected to subsequent analysis; LGCs transfected with the empty vector (pEX-4) and negative control siRNA served as negative controls (pEX-4-Control and NC respectively). Meanwhile, as preliminary experiments, we compared the pEX-4-VDR and siRNA-393 treatments not only with their negative controls (empty vector (pEX-4) and negative control siRNA) but also with blank controls and found no significant differences in VDR gene and protein levels between the blank and negative controls. Therefore, in the final study, we used negative controls to observe the effects of VDR overexpression and knockdown. Moreover, an appropriate concentration of Vit D_3 (as determined in the first experiment) was added to the VDR overexpression or silencing groups.

Immunohistochemistry and immunofluorescent assays

Immunohistochemical analysis was performed according to our previously described method (Yao *et al.* 2017*a*). Briefly, whole fixed ovaries were sectioned into 6-μm slices, the endogenous peroxidase was quenched by incubating the deparaffinised sections with 3% H_2O_2 at 37°C for 10 min and antigen retrieval was carried out in citrate buffer solution at 100°C for 15 min. After the sections were blocked with 5% bovine serum albumin (BSA) at 37°C for 30 min, they were incubated with rabbit anti-VDR antibody overnight at 4°C and further incubated with goat anti-rabbit IgG at 37°C for 30 min. Negative controls were treated with Tris-buffered saline (TBS) buffer instead of primary antibody. The sections were next treated with 3,3'-diaminobenzidine (DAB) and examined under a microscope (Nikon).

Immunofluorescence assays were performed according to our previously described method (Yao *et al.* 2018), with a slight modification. In brief, LGCs were seeded onto coverslips and cultured in 24-well plates (5×10^4 cells per well) for 48 h, as described above for the first experiment. The LGCs were then fixed with 4% paraformaldehyde for 20 min, permeabilised with 0.25% Triton X-100 for 10 min, blocked with 5% BSA for 1 h at room temperature (about 25°C) , incubated with rabbit anti-VDR antibody overnight at 4°C and further incubated with goat anti-rabbit conjugated with Alexa Fluor 594 for 1 h at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and the cells were examined under an LSM710 laser-scanning confocal microscope (Carl Zeiss). Negative control images (NCI) were treated with TBS instead of primary antibody.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

To assess cell viability, an MTT assay (Boster Co., Ltd) was performed. A total of 5×10^3 cells per well in 100 μL fresh BCM were seeded in 96-well plates for 24 h. The *in vitro* culture was carried out as described in the subsection 'Experimental design of *in vitro* LGC culture.' After 48 h, 10 μL of the MTT reagent was added to 100 μL of the supernatant from each well and incubated in a humidified atmosphere containing 5% CO_2 at 37°C for 4 h. Subsequently, the medium was removed and 100 μL of a formazan solubilisation solution was added to dissolve the formazan crystals. Optical density was measured at 570 nm on a Synergy H1/H1MFD analyser (BD Biosciences).

5′-ethynyl-2′-deoxyuridine (EdU) incorporation assay

Cell proliferation assays were performed using the Click-iT EdU assay kit (KeyGen Biotech Co., Ltd). Towards this end, 5×10^4 cells in 100 μL fresh BCM were seeded per well in 24-well plates for 24 h. The *in vitro* culture was carried out as described above for the second experiment. After transfection at 48 h, 200 μL of an EdU solution was added at a final concentration of 50 μM and was allowed to react with the cells for 2 h. The cells were then washed with DPBS three times, fixed with 4% formaldehyde for 30 min and permeabilised with 0.5% Triton X-100 for 10 min at room temperature (about 25°C). Next, the cells were incubated with 200 μL Click-iT reaction buffer for 30 min and washed with DPBS containing 3% BSA. DNA was stained with Hoechst 33342 for 30 min and the results were visualised by means of an LSM710 laser-scanning confocal microscope (- Carl Zeiss). NCI were treated with an equal

Table 3. Primer sequences used for this study

Gene	Primer sequence (5′–3′)	Genebank No.	Size (bp)
VDR	F: TCCTCTCCAGACACAACGGA R: ACAGGTCCAGGGTCACAGAA	XM_018047873.1	91
p21	F: CTAAGTGGGCAAATATGGGTCTGG R: CAGGATGCTACAGGAGCTGGAAG	XM_018039118.1	107
p27	F: AAACCCAGAGGACACGCATT R: GGCAGGTCGCTTCCTTATCC	XM_005680816.3	100
CDK4	F: CGTTGGCTGTATCTTTGC R: GATTCGCTTGTGTGGGTT	XM_005680266.3	256
cyclin D1	F: CCGTCCATGCGGAAGATC R: CAGGAAGCGGTCCAGGTAG	XM_018043271.1	108
BAX	F: GCATCCACCAAGAAGCTGAG R: CCGCCACTCGGAAAAAGAC	XM_013971446.2	130
Bcl-2	F: ATGTGTGTGGAGAGCGTCA R: AGAGACAGCCAGGAGAAATC	XM_018039337.1	254
GAPDH	F: CGACTTCAACAGCGACACTCAC R: CCCTGTTGCTGTAGCCGAATTC	XM_005680968.3	119

volume of culture medium without FBS instead of EdU. The percentage of EdU-positive cells was calculated from five different fields by semiquantitative analysis.

Flow cytometric analysis of cell cycle

This procedure was performed as described by Fang *et al.* (2017) with minor modifications. Briefly, cells (with silenced or overexpressed VDR) at ~90% confluence were digested, washed and resuspended in DPBS. The cells were then fixed with cold 70% ethanol overnight at −20°C, washed with cold DPBS three times, incubated with 100 µL RNase A (10 ng mL^{-1}) for 30 min at 37°C and finally stained with 1 mL propidium iodide for 30 min. The cell cycle stages were identified by flow cytometry (BD Biosciences).

qRT-PCR

RNA extraction and cDNA synthesis were performed according to our previously described method (Yao *et al.* 2017*b*). Briefly, total RNA was extracted from the collected follicles and LGCs using Trizol Reagent (Invitrogen). RNA concentration and purity were detected using an ND-2000 spectrophotometer (NanoDrop). Next, cDNA was synthesised using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara). The qRT-PCR primers were designed using Primer 5.0 software (Premier, Canada) and are presented in Table 3. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as an internal control. An ABI 7500 real-time PCR system (Applied Biosystems) was used for qRT-PCR and the reactions were carried out using the FastStart SYBR Green Master Mix (Roche) according to the manufacturer's protocol. The relative mRNA levels of target genes were analysed using the $^{\Delta\Delta}$CT method.

Western blotting

Total-protein samples were prepared from the collected follicles and LGCs using the cell protein extraction reagent (Beyotime Biotechnology) containing phenylmethanesulfonyl fluoride. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology).

Protein from each group (20–40 µg) was separated by electrophoresis on a 12% sodium dodecyl sulphate (SDS) polyacrylamide gel and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). After blocking with 5% (w/v) fat-free milk for 1 h at room temperature, the membranes were incubated with anti-VDR, anti-p27, anti-B-cell lymphoma 2 (Bcl-2), anti-Bcl-2-associated X protein (BAX) or anti-β-actin antibodies overnight at 4°C, followed by incubation with a goat anti-rabbit IgG antibody for 1 h at room temperature. The proteins were visualised using an enhanced chemiluminescence detection system (Fujifilm) and the chemiluminescence intensity of each protein band was quantified by the Image J software (National Institutes of Health).

Statistical analysis

All the experiments were conducted three times independently and each experiment involved five replicates; the data are expressed as the mean ± standard error of the mean. Statistical analysis was carried out as follows: (1) two-way analysis of variance (ANOVA) with the follicle state as one variable and size of the follicle as the other variable was performed to study their interaction, (2) data on the follicles of different diameters and exposure of LGCs to different concentrations of Vit D_3 were evaluated by one-way ANOVA and (3) other data were subjected to Tukey's test. All analyses were performed using the SPSS Statistics software (Version 19.0; IBM Corp.) and $P < 0.05$ was considered to be statistically significant.

Results

Expression patterns of VDR in healthy and atretic follicles of different sizes

VDR was localised on the GCs and theca cells (TCs) in healthy and atretic follicles (Fig. 1*a*, *b*). No positive signal was detected in the negative controls (Fig. 1*c*). There was no significant

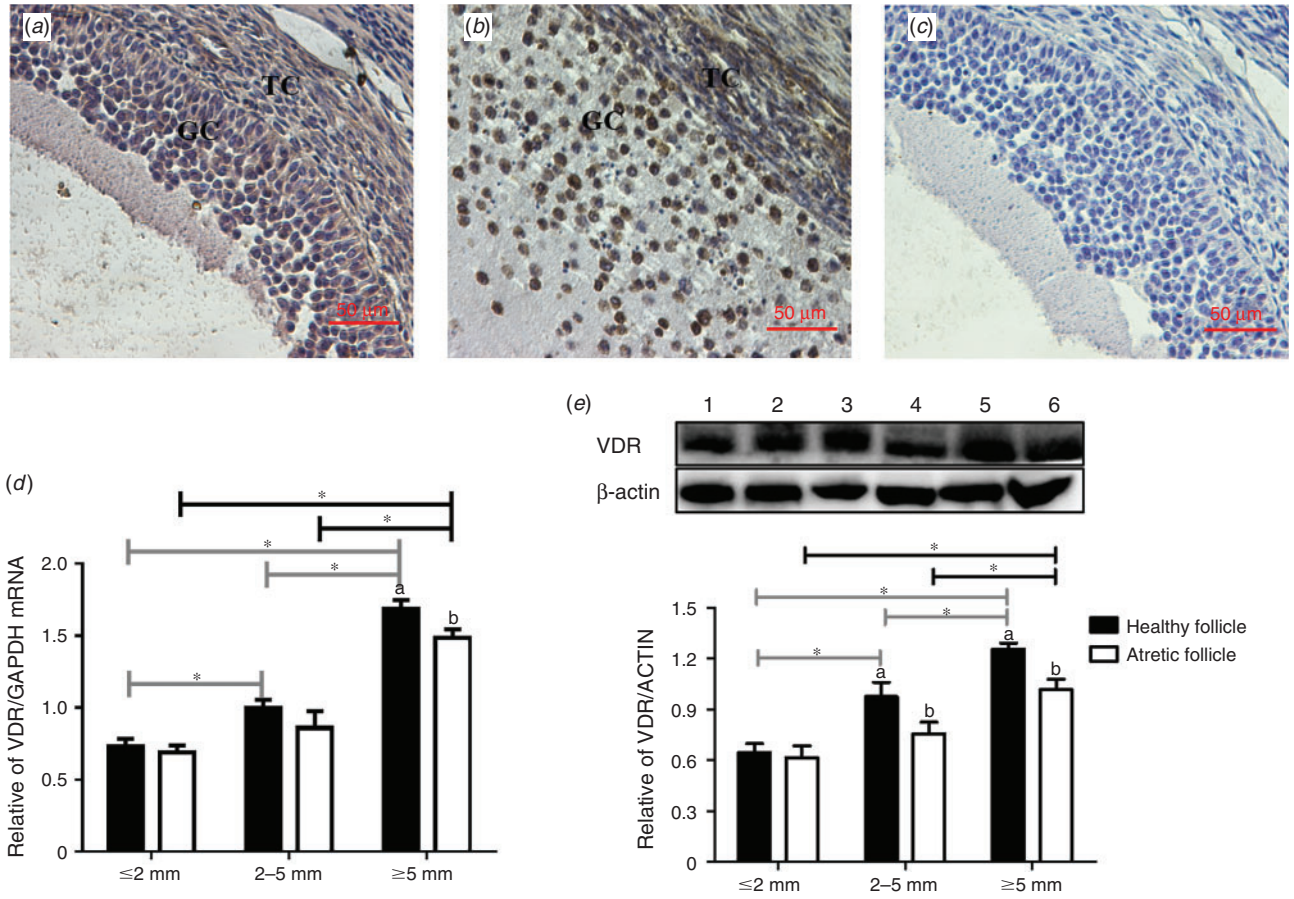

Fig. 1. VDR is expressed in both healthy follicles and atretic follicles. (*a–c*) Immunohistochemical staining of VDR in goat ovaries. (*d*) qRT-PCR and (*e*) western blot analyses were performed to detect VDR expression in healthy follicles or atretic follicles of different sizes (follicle diameter ≤2 mm, 2–5 mm and ≥5 mm). Data are presented as mean ± s.e.m. Different lower-case, superscript letters indicate that values are significantly different between healthy and atretic follicles ($P < 0.05$). In the same follicle state, asterisks (*) indicate significant difference between different follicle diameters (≤2, 2–5 and ≥5 mm; $P < 0.05$); grey asterisks represent healthy follicle group and black asterisks represent atretic follicle group. GC, granulosa cell; TC, theca cell. Scale bars = 50 μm.

interaction between the follicle size and state in terms of VDR mRNA ($P < 0.511$) and protein ($P < 0.245$) expression. As shown in Fig. 1*d* and *e*, VDR mRNA and protein levels increased significantly with increasing follicle size ($P < 0.05$). Among the ≥5 mm follicles, the expression of VDR was significantly higher in the healthy follicles than in the atretic follicles ($P < 0.05$). However, no significant difference was observed between the ≤2 mm healthy and atretic follicles ($P > 0.05$). In addition, VDR mRNA expression among the 2–5 mm healthy and atretic follicles showed no significant difference ($P > 0.05$); however, a significant difference in VDR protein levels was observed ($P < 0.05$).

Vit D_3 promotes proliferation of goat LGCs cultured in vitro

After treatment of LGCs with various concentrations of Vit D_3 for 48 h, the expression of VDR was determined in all the groups (Fig. 2*a*). The intensity of the red fluorescence of VDR in the Vit D_3-treated groups was significantly higher than in the control group and the highest level was detected in the 10 nM Vit D_3-treated group (Fig. 2*b*; $P < 0.05$). A similar trend was observed in the MTT assay (Fig. 2*c*). However, there was no significant difference between the 1 nM and 100 nM Vit D_3-treated groups (Fig. 2*c*; $P > 0.05$).

As presented in Fig. 3, the mRNA expression of cyclin-dependent kinase 4 (*CDK4*) and *cyclin D1* in the Vit D_3-treated groups was significantly higher than in the control group and the highest mRNA expression among these was found in the 10 nM group ($P < 0.05$). Furthermore, the mRNA expression of *CDK4* and *cyclin D1* in the 1 nM group was significantly higher than in the 100 nM group ($P < 0.05$). However, no significant difference in *cyclin D1* mRNA expression was observed between the 1 nM and 10 nM groups ($P > 0.05$). The highest and the lowest mRNA expression of *p21* was found in the control group and 10 nM group respectively ($P < 0.05$). The *p21* mRNA level in the 100 nM group was significantly higher than in the 1 nM and 10 nM groups ($P < 0.05$).

Efficiency of VDR overexpression and suppression in goat LGCs

To overexpress VDR, the pEX-4-VDR plasmid was constructed by cloning VDR into the pEX-4 vector (Fig. 4*a*) and was verified

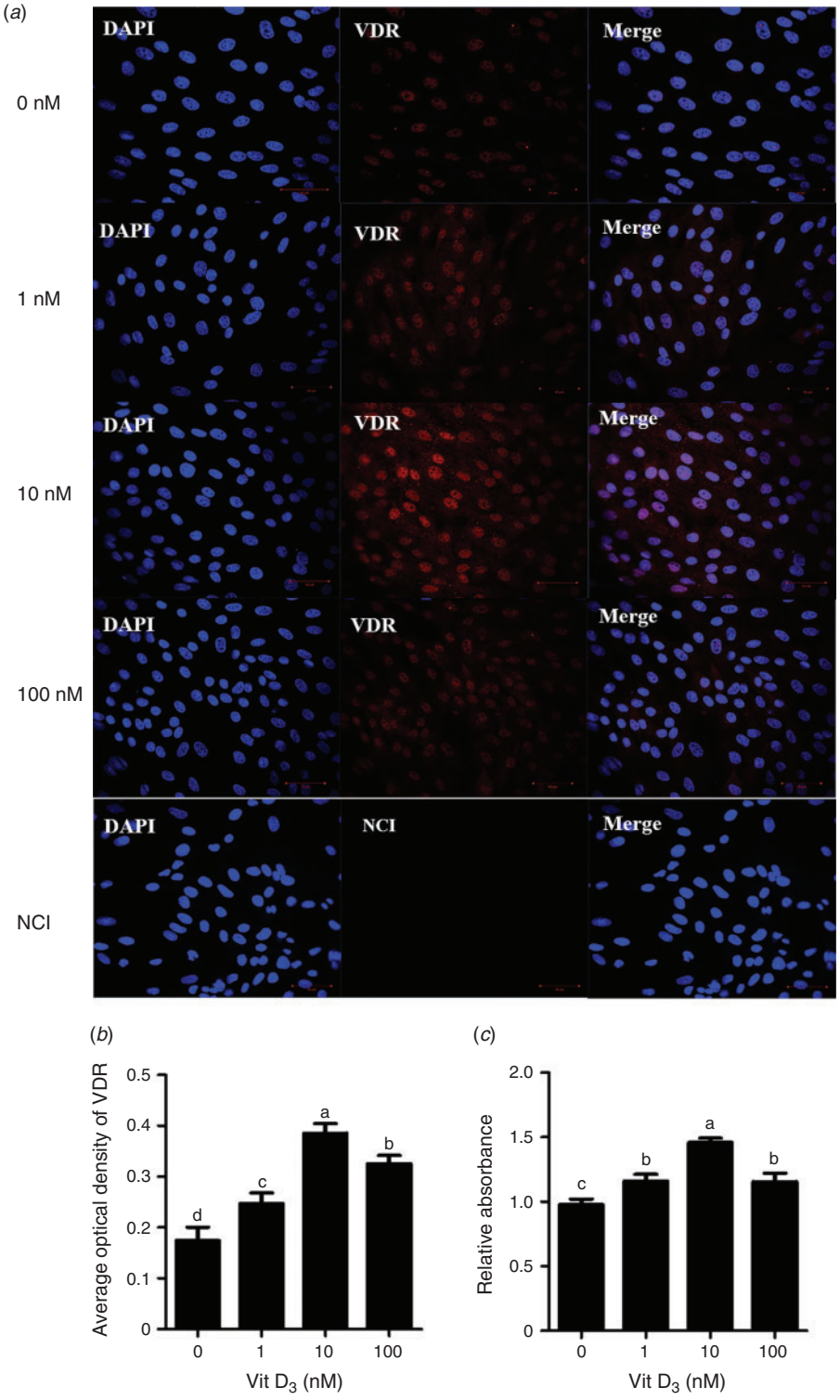

Fig. 2. *In vitro* supplementation with Vit D_3 stimulated goat LGC proliferation. (*a*) Immunofluorescence assay for VDR in various treatment groups after 48 h of culture. (*b*) Average optical densities for VDR expression in different treatment groups. (*c*) The absorbance at 570 nm for MTT assay was used to assess LGC proliferation. Data are presented as mean ± s.e.m. and different superscript letters (a–d) represent significant differences ($P < 0.05$). Scale bars = 50 μm.

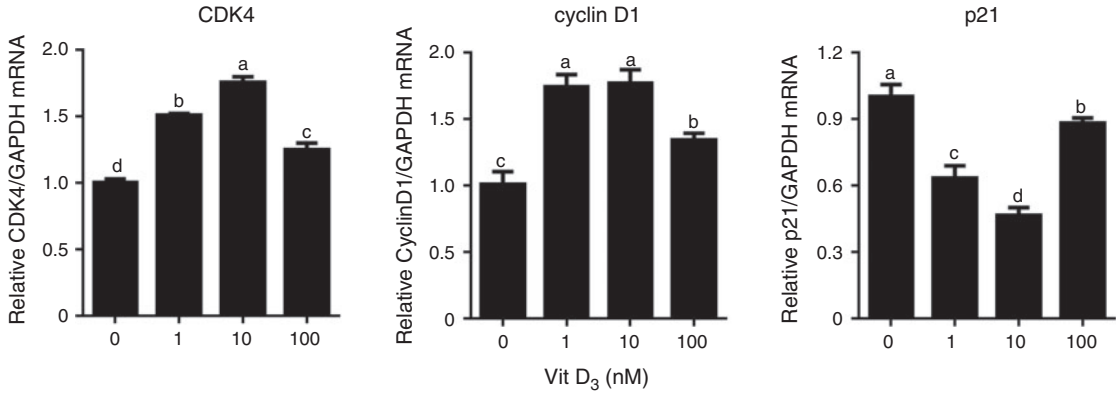

Fig. 3. Vit D_3 altered the mRNA expression of cell cycle-related genes (*CDK4*, *cyclin D1* and *p21*) in LGCs. Data are presented as mean ± s.e.m. and different superscript letters (a–d) represent significant differences ($P < 0.05$).

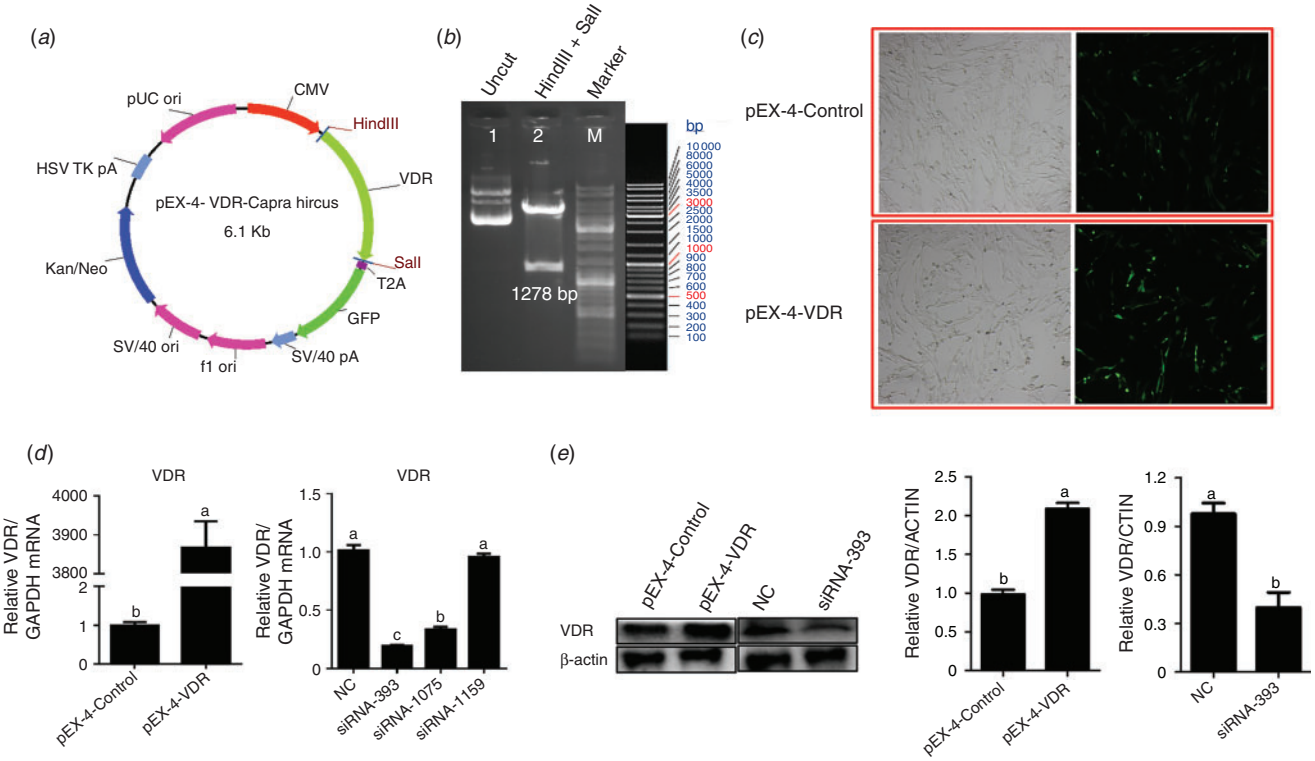

Fig. 4. Appraisal of VDR overexpression and knockdown efficiency in goat LGCs. (*a*) Backbone of the VDR overexpression vector. (*b*) Restriction enzyme digestion of the pEX-4-VDR plasmid. (*c*) Representative micrographs of overexpression vector transfected into goat LGCs. (*d–e*) Overexpression efficiency and suppression efficiency were evaluated by (*d*) qRT-PCR and (*e*) western blot assays. Data are presented as mean ± s.e.m. and different superscript letters (a–c) represent significant differences ($P < 0.05$).

by cleaving it into 4773- and 1278-bp fragments using *HindIII* and *SalI* (Fig. 4*b*). At 48 h after the transfection, green fluorescence was observed in both the control group and VDR-transfected group (Fig. 4*c*). However, the mRNA and protein expression of VDR in the overexpression group was significantly higher than in the control group (Fig. 4*d* and *e* respectively; $P < 0.05$). Next, siRNA was employed for the suppression of VDR in LGCs. The knockdown efficiency of siRNA-393 was the highest among the three siRNAs tested (Fig. 4*d*) and the mRNA and protein expression levels in the siRNA-393 group were significantly lower than in the negative control group (Fig. 4*d* and *e* respectively; $P < 0.05$).

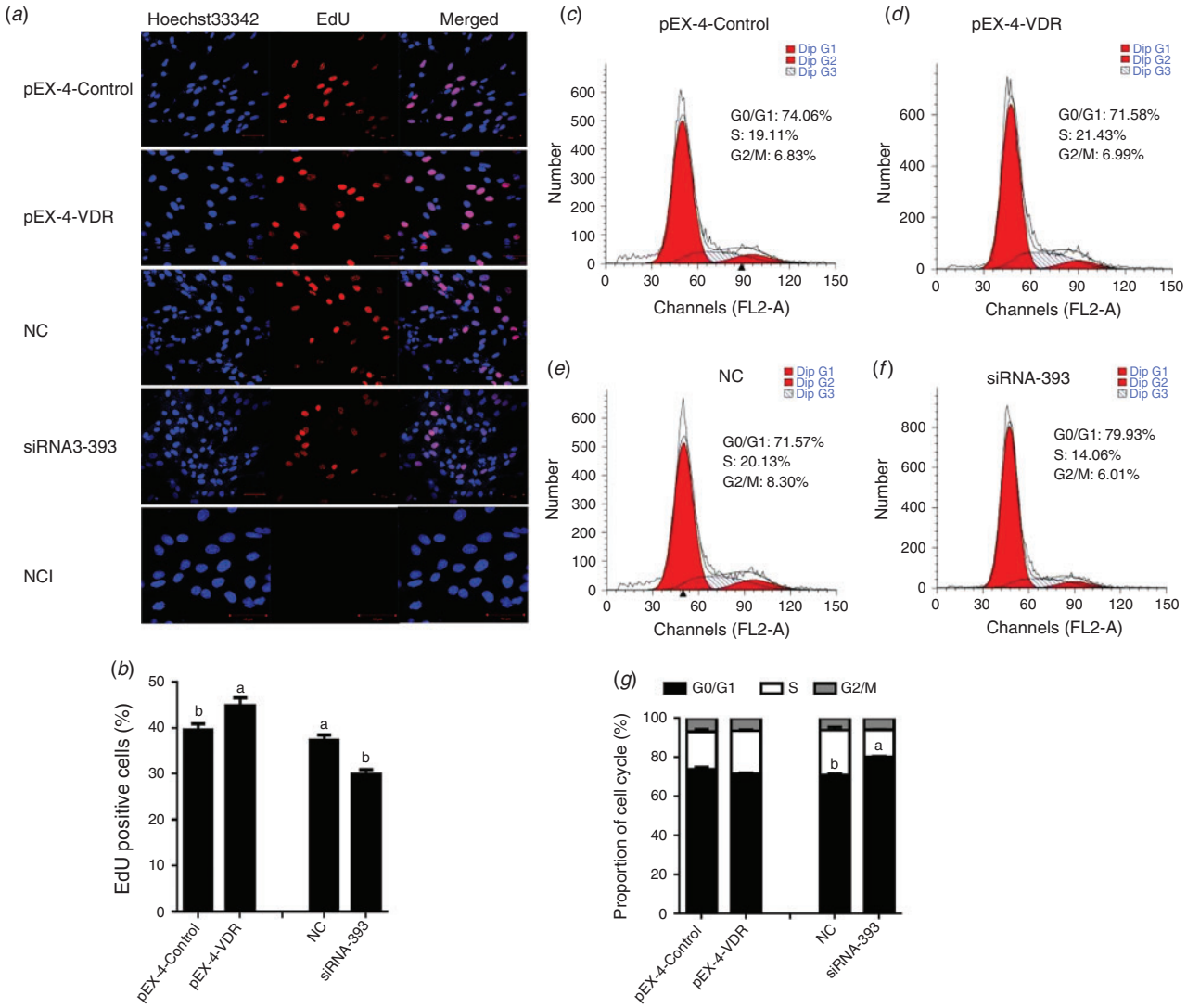

Fig. 5. Both VDR overexpression and knockdown influenced the cell proliferation and cell cycle distribution of goat LGCs. (*a*) Representative micrographs and (*b*) quantification of EdU-incorporating cells among LGCs are shown. (*c–f*) The cell cycle distributions in goat LGCs with VDR (*c–d*) overexpression and (*e–f*) silencing were analysed by flow cytometry. (*g*) The histogram presents the percentages of LGCs in different phases of the cell cycle. Data are given as mean ± s.e.m. and different superscript letters (a, b) denote significant differences ($P < 0.05$). Scale bars = 50 μm.

Influence of VDR on proliferation and cell cycle distribution of goat LGCs

To assess the effect of ectopic expression of VDR on cell proliferation, a Click-iT EdU assay was performed (Fig. 5*a*, *b*). Overexpression of VDR promoted, whereas its knockdown inhibited cell proliferation (Fig. 5*b*; $P < 0.05$). Furthermore, flow cytometric analysis revealed that the percentage of G0/G1 phase cells significantly increased and that of S phase cells decreased in the VDR knockdown group (Fig. 5*e–g*; $P < 0.05$). However, overexpression of VDR did not change the percentage of G0/G1 or S phase cells (Fig. 5*c*, *d*, *g*; $P > 0.05$).

Expression of cell cycle- (p21, p27, CDK4 and cyclin D1) and apoptosis- (BAX and Bcl-2) related genes was analysed by qRT-PCR and western blotting (Fig. 6). VDR suppression significantly decreased *CDK4*, *cyclin D1* and *Bcl-2* mRNA expression (Fig. 6*a*, *b*, *e*; $P < 0.05$), but increased that of *p21* and *BAX* as well as the BAX/Bcl-2 ratio (Fig. 6*c*, *d*, *f*; $P < 0.05$). However, mRNA expression of *CDK4*, *cyclin D1* and *p21* in the VDR overexpression group showed a trend opposite to that in the VDR knockdown group (Fig. 6*a–c*; $P < 0.05$). No effects on *BAX* and *Bcl-2* mRNA expression or on the BAX/Bcl-2 ratio were observed in the overexpression group (Fig. 6*d–f*; $P > 0.05$). However, overexpression of VDR significantly decreased the protein level of BAX and the BAX/Bcl-2 ratio and increased the level of Bcl-2 protein (Fig. 6*h*, *j–l*; $P < 0.05$). Although there were no significant changes in BAX and Bcl-2 protein expression in the knockdown group (Fig. 6*h*, *j*, *k*; $P > 0.05$), the change in the BAX/Bcl-2 ratio was statistically significant (Fig. 6*l*; $P < 0.05$). Both mRNA and protein

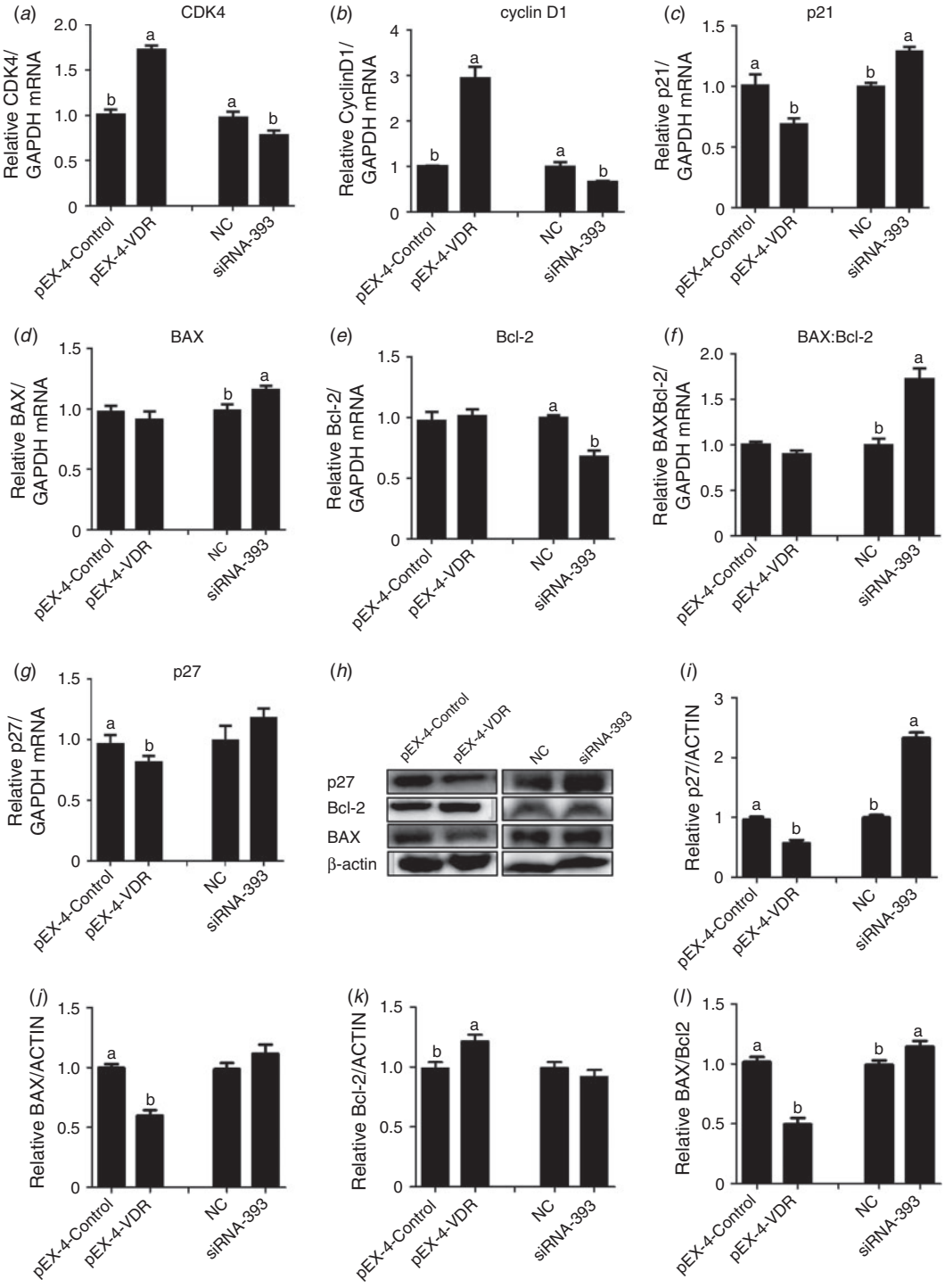

Fig. 6. Overexpression and suppression of VDR altered the (*a*–*g*) mRNA and (*h*–*l*) protein expression levels of cell cycle-related (CDK4, cyclin D1, p21 and p27) and apoptosis-related (BAX and Bcl-2) genes in goat LGCs. Data are presented as mean ± s.e.m. and different superscript letters (a, b) represent significant differences ($P < 0.05$).

expression levels of p27 decreased in the overexpression group (Fig. 6*g*–*i*; $P < 0.05$). In addition, only the protein expression of p27 in the VDR knockdown group was significantly greater than in the control group (Fig. 6*h*, *i*; $P < 0.05$).

Vit D_3 partially reinforces the effects of VDR overexpression on LGC proliferation

As depicted in Fig. 7*a*, overexpression of VDR was significantly associated with increased proliferation of LGCs in the presence

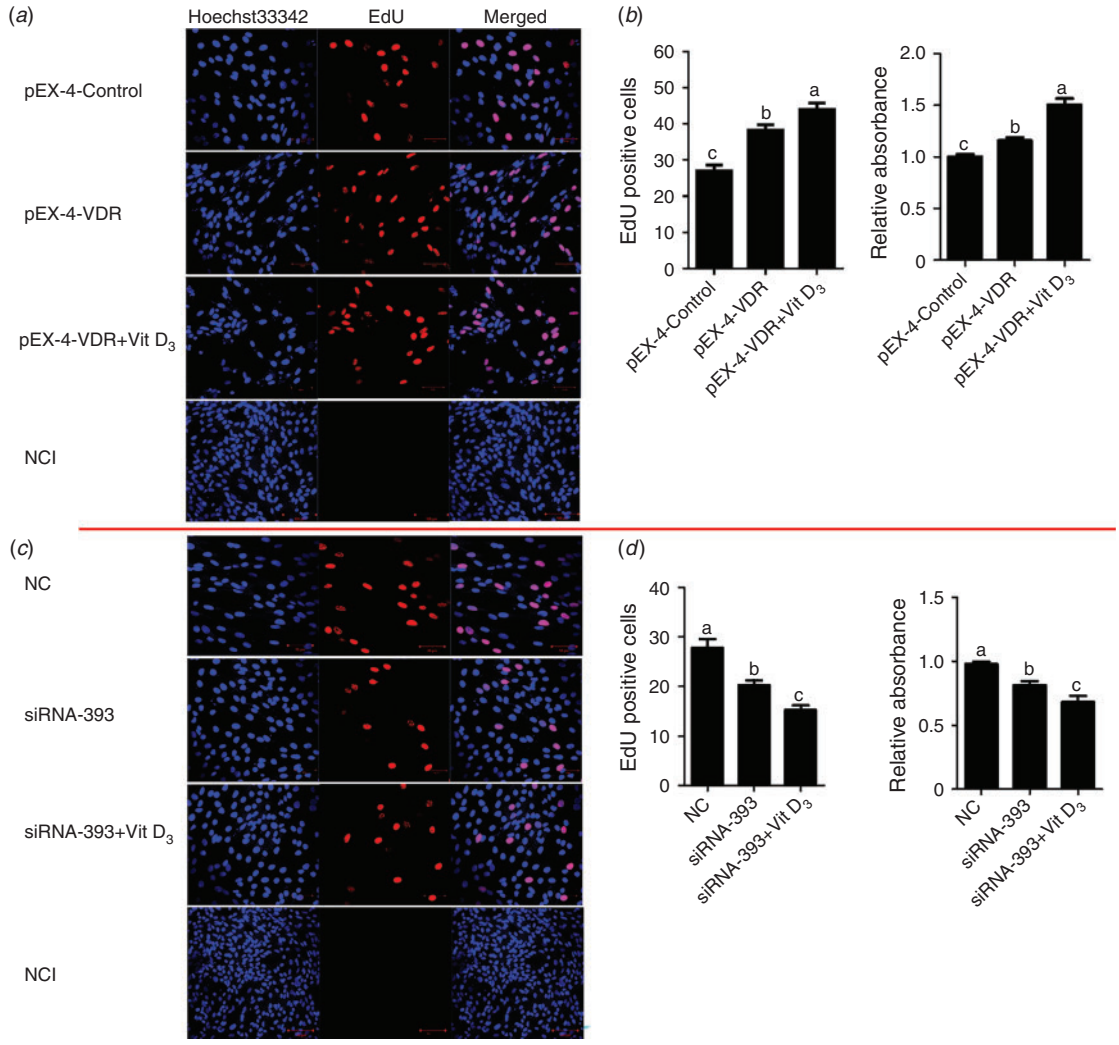

Fig. 7. Vit D_3 enhanced proliferation of goat LGCs overexpressing VDR but decreased proliferation of goat LGCs with VDR knockdown. Proliferation of goat LGCs was analysed by (*a*, *c*) EdU and (*b*, *d*) MTT assays in different treatment groups. Data are presented as mean ± s.e.m. and different superscript letters (a–c) represent significant differences ($P < 0.05$). Scale bars = 50 μm.

of Vit D_3 than in its absence ($P < 0.05$), as supported by the MTT assay results (Fig. 7*b*; $P < 0.05$). In contrast, Vit D_3 significantly inhibited LGC proliferation after VDR knockdown by siRNA (Fig. 7*c*; $P < 0.05$). A similar trend was observed in the MTT assay (Fig. 7*d*; $P > 0.05$).

Discussion

Accumulating evidence indicates that Vit D_3 performs important functions in both male and female reproduction, primarily via VDR (Boisen *et al.* 2017; Lorenzen *et al.* 2017). Initially, we demonstrated that VDR is mainly localised in GCs and TCs and its expression increases with increasing follicle size regardless of the state of the follicles. This finding agrees with the results of other studies (Wojtusik and Johnson 2012; Yao *et al.* 2017*b*). In the present study, the expression of VDR was significantly higher in healthy follicles than in atretic follicles when the follicle size was ≥2 mm; this finding suggests that Vit D_3 may be important for follicular development.

According to some reports, including a study from our laboratory, an appropriate concentration of Vit D_3 can affect follicle development by promoting GC proliferation and steroidogenesis (Merhi *et al.* 2014; Hong *et al.* 2017; Yao *et al.* 2017*b*). However, whether Vit D_3 has a dose-dependent effect on *in vitro* proliferation of goat GCs remained unclear. We observed that among various concentrations of Vit D_3, the 10 nM dose induced the highest proliferation rate of cultured goat LGCs; this rate decreased as the Vit D_3 concentration increased to 100 nM. These findings suggest that Vit D_3 enhances the proliferation of LGCs in a dose-dependent manner. This observation is supported by another study, which revealed that Vit D_3 enhances the proliferation of hen GCs in a dose-dependent manner (Wojtusik and Johnson 2012). Additionally, enhanced proliferation of endothelial progenitor cells with 10 nM Vit D_3 has been reported (Grundmann *et al.* 2012).

Although our findings were corroborated by both immunofluorescence and cell proliferation assays, some studies indicate that 10 nM Vit D_3 inhibits cell proliferation (Olsson *et al.* 2016; Chen *et al.* 2018*a*; Emanuelsson *et al.* 2018). This discrepancy might be related to differences in the requirements for Vit D_3 by different cell types. In addition, we observed a positive effect of Vit D_3 addition on VDR expression, which is corroborated by previous reports (Yao *et al.* 2017*b*; Xu *et al.* 2018). However, a combination of Vit D_3 at 100 nM and testosterone did not affect VDR levels in rat GCs (Lee *et al.* 2014), the details of which need to be further studied.

The molecular mechanisms by which Vit D_3 regulates the proliferation of LGCs are still not fully understood. Cell proliferation is precisely controlled by cyclins and cyclin-dependent kinases (CDKs), whereas CDK function is tightly regulated by cyclin-dependent kinase inhibitors (CKIs), such as p21 (Stanley *et al.* 2011). Moreover, the activation of cyclin D1–CDK4 complexes is required for cells to reach the G1/S restriction point (Cong *et al.* 2017). We previously reported that Vit D_3 drastically increases the percentage of cells in the S phase while decreasing that of cells in the G0/G1 phase (Yao *et al.* 2017*b*). This concept fits with our present results, where Vit D_3 supplementation ($\leq$10 nM) increased the mRNA expression of *CDK4* and *cyclin D1* and decreased that of *p21*. Consistent with earlier reports (Verlinden *et al.* 1998; Yao *et al.* 2017*b*), Vit D_3 promoted progression of the cell cycle from the G0/G1 to the S phase through upregulation of *CDK4* and *cyclin D1* and downregulation of *p21*. Moreover, excess Vit D_3 (e.g. 100 nM) restricts cell proliferation, as evidenced by the MTT results, by downregulating the expression of *CDK4* and *cyclin D1* and upregulating *p21* compared with the other Vit D_3-treated groups (Chiang *et al.* 2016, 2017).

VDR may play vital roles in proliferation and apoptosis in many cell types (Consiglio *et al.* 2014; He *et al.* 2018; Oda *et al.* 2018; Ricca *et al.* 2018), including acting as a the unaligned effects (Alimirah *et al.* 2010). Although the ligand-independent actions of VDR have been widely confirmed using many cell models, its signal activator has not been identified (Pike *et al.* 2017). Such independent effects of VDR could be partially responsible for the repression and induction of goat LGC proliferation upon VDR knockdown and overexpression respectively. To some extent, this theory could explain the lower expression of VDR in atretic follicles than in healthy follicles. On the contrary, some studies have reported a negative correlation between VDR levels and proliferation in cancer cells (Koike *et al.* 1997; Kovalenko *et al.* 2011; Chen *et al.* 2018*b*). This discrepancy may be attributed to the difference in cell types and conditions, since normal cells have higher VDR expression than cancer cells (Koike *et al.* 1997).

To evaluate the specific effects of VDR knockdown or overexpression, goat LGCs were further treated with an optimum concentration of Vit D_3 (10 nM). The different proliferation responses to Vit D_3 addition suggest that Vit D_3 elicits specific responses with respect to cell proliferation depending on the expression level of VDR. Likewise, although VDR has significant ligand-independent effects, the availability of Vit D_3 could efficiently regulate its reactions. Notably, the inhibitory effect of Vit D_3 supplementation with VDR knockdown observed in our study could mimic the anti-proliferative effect of Vit D_3 in the case of cancer cells that weakly express VDR (Koike *et al.* 1997).

To investigate the mechanism by which VDR regulates the proliferation and apoptosis of LGCs, we attempted to identify the downstream targets of VDR in goat LGCs. First, the cell cycle distribution results demonstrated that the ectopic expression of VDR in LGCs changed the percentages of LGCs in the G0/G1 and S phases of the cell cycle. Similar results were also previously reported (Consiglio *et al.* 2014). Second, analysis of cell cycle-related genes revealed that the expression of *CDK4* and *cyclin D1* was low in the VDR knockdown group, whereas the reverse trend was observed in the VDR overexpression group. Both p21 and p27 interact with cyclins to inhibit the biological activity of cyclin–CDK complexes and to retard the G1-to-S-phase progression of the cell cycle (Orlando *et al.* 2015). In the present study, the expression of *p21* and *p27* was significantly decreased in the VDR overexpression group and increased in the knockdown group. *p21* has been considered as a primary anti-proliferation target for VDR in the presence of Vit D_3 (Rao *et al.* 2004; Saramäki *et al.* 2006). Taken together, our results suggest that VDR directly modulates the proliferation of LGCs by regulating the expression of cell cycle-related genes.

Apoptosis is a precisely controlled process regulated by members of the caspase and Bcl-2 families (Alabsi *et al.* 2016; Sahin *et al.* 2018). In the present study, VDR knockdown significantly increased BAX expression and decreased Bcl-2 expression, suggesting that VDR silencing can induce apoptosis of LGCs. Meanwhile, we observed anti-apoptotic effects of VDR overexpression only at the protein level, which could indicate the regulatory effect of VDR on the transcription of apoptotic genes. Furthermore, VDR could interact with other lipid ligands and its functions reflect a potential unliganded effect to repress the transcription of some genes (Dowd and MacDonald 2010). Additionally, the potential of transcription-regulatory function of ligand-independent VDR was also noted *in vivo* (Lee and Pike 2016). Notably, the unliganded activity of VDR effectively impairs calcium homeostasis (Skorija *et al.* 2005; Huet *et al.* 2015). Therefore, although VDR has significant ligand-independent effects, the availability of Vit D_3 could efficiently regulate these. Extensive research has shown that in cancer cells VDR regulates proliferation and apoptosis by controlling the expression of- transient receptor potential cation channel, subfamily V, member 5 (TRPV5; Chen *et al.* 2018*b*) and tumour necrosis factor α (TNF-α; Zhang *et al.* 2014). Further studies are needed to elucidate the exact mechanism of the Vit D_3–VDR interaction in goat LGCs.

Culturing of GCs is a useful tool to understand the molecular processes of follicle development; nevertheless, suitable *in vitro* models to explore ovarian functions are limited (Skory *et al.* 2015). In this study, we used LGCs as a model for exploring the potential dose-dependent effects of Vit D_3 on the proliferation and apoptosis of goat GCs *in vitro* and for elucidating the underlying mechanism by means of overexpressing and silencing VDR, since the cultured GCs are probably converted into LGCs during *in vitro* culture (Tosca *et al.* 2010). Although this model may not be ideal for GC studies, our previous study (Yao *et al.* 2017*b*) and other reports (Smolikova *et al.* 2013;

Merhi *et al.* 2014; Hong *et al.* 2017; Merhi *et al.* 2018) have shown that LGCs are responsive to Vit D_3 treatment *in vitro*.

In conclusion, this study indicates that Vit D_3 affects proliferation of goat LGCs in a dose-dependent manner by regulating the expression of cell cycle-related genes. The highest proliferation rate was obtained at 10 nM Vit D_3 supplementation, which may serve as a reference point for calculating Vit D_3 requirements in non-pregnant ewes. Moreover, since apoptosis of GCs is considered to be the main mechanism underlying follicular atresia (Asselin *et al.* 2000), VDR could be involved in the regulation of the follicular atresia process in particular, by affecting the transcription of apoptosis-related genes. We also suggest that the expression patterns of VDR in GCs may dominate follicular development by regulating proliferation- and apoptosis-related genes, considering that follicular development involves accurately controlled and timed processes that include many endocrine and paracrine functions (Richards 2018; Wirleitner *et al.* 2018). Finally, VDR availability could direct the cell proliferation responses to Vit D_3. Further studies are needed to better understand the molecular mechanisms underlying the action of Vit D_3 and VDR on follicular development.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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