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Vitamin D receptor expression and potential role of vitamin D on cell proliferation and steroidogenesis in goat ovarian granulosa cells

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ABSTRACT

This study aimed to investigate the expression of the vitamin D receptor (VDR) in goat follicles and to determine the effects of Vit D_3 supplementation on goat granulosa cells (GCs) function linked to follicular development. The results demonstrated that VDR was prominently localized in GCs, with expression increasing with follicle diameter. Addition of Vit D_3 (1α,25-$(OH)_2VD_3$; 10 nM) to GCs caused an increase in VDR and in steroidogenic acute regulator (*StAR*) and 3β-hydroxysteroid dehydrogenase (*3β-HSD*) mRNA expression. Additionally, Vit D_3 increased the cyclic adenosine monophosphate (cAMP), estradiol (E_2), and progesterone (P_4) levels, while it decreased anti-müllerian hormone receptor (*AMHR*) and follicle-stimulating hormone receptor (*FSHR*) mRNA expression ($P < 0.05$). Addition of FSH remarkably increased E_2, P_4, and cAMP levels ($P < 0.05$), and Vit D_3 further enhanced the E_2 and cAMP levels in the presence of FSH ($P < 0.05$). Vit D_3 significantly induced the mRNA expression of *CDK4* and *CyclinD1*, and downregulated *P21* gene expression ($P < 0.05$). In addition, Vit D_3 significantly decreased reactive oxygen species (ROS) production and increased the mRNA and protein expression of superoxide dismutase 2 (SOD2) and catalase (CAT) ($P < 0.05$). In conclusion, VDR is expressed in GCs of the goat ovaries and Vit D_3 might play an important role in GCs proliferation by regulating cellular oxidative stress and cell cycle-related genes. Meanwhile, Vit D_3 enhances the E_2 and P_4 output of GCs by regulating the expression of *3β-HSD* and *StAR* and the level of cAMP, which regulate steroidogenesis, supporting a potential role for Vit D_3 in follicular development.

1. Introduction

The active form of Vit D_3 (1α,25-$(OH)_2VD_3$) is a lipid-soluble secosteroid hormone that has been established to play pleiotropic roles in a wide spectrum of biological functions [1]. Vit D_3 activity is mediated via the vitamin D receptor (VDR), which is a member of the nuclear steroid hormone receptor superfamily [2] and is expressed in multiple tissues. VDR is expressed in female reproductive organs, including the placenta [3,4], uterus [5,6], and ovary [3,5,7], in humans and rodents. VDR has been also detected in testes [8], epididymis [8], and spermatids [9]. VDR null-mutant male mice display gonadal insufficiency; however, estradiol (E_2) supplementation restores normal function in the testes [10]. These findings suggest that Vit D_3 plays an important role in reproductive organs [11]. To date, most of the research on VDR in follicular development has focused on humans and rodents, while its expression pattern in goat follicular development has never been reported.

Many studies have demonstrated that Vit D_3 has related to reproductive physiological mechanisms [12]. Vit D_3 deficiency in humans and rodents has been associated with various disorders, such as infertility [13] and ovulatory dysfunction [14]. Our previous studies demonstrated that VDR is expressed in Leydig cells [8] and that Vit D_3 could increase testosterone secretion and mitochondrial dehydrogenase activity in Leydig cells *in vitro* [15]. The presence of VDR in ovarian granulosa cells (GCs) [16,17] suggests that Vit D_3 may be associated with steroid hormone synthesis and secretion. Reports by Merhi et al. [17] and Smolikova et al. [18] supported that Vit D_3 is related to steroidogenesis in human and porcine GCs; however, the mechanism is still not well understood. Moreover, to our knowledge, direct effects of Vit D_3 on basal and follicle-stimulating hormone (FSH)-induced GCs steroid hormone synthesis and secretion have not been reported.

Most follicles undergo atresia, which is attributed to the

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apoptosis of GCs [19,20]. Hence, inhibition of GCs apoptosis is important for promoting follicular development. Accumulating evidence indicates that Vit D_3 can modulate cell proliferation (e.g., of skeletal muscle cells, glomerular mesangial cells, and cancer cells) and/or apoptosis through regulating the expression of cell cycle- and apoptosis-related genes [21,22]. In addition, Vit D_3 can reduce tissue and DNA damage by inhibiting apoptosis through modulating antioxidant enzyme activities and scavenging reactive oxygen species (ROS) [23–25]. While these studies demonstrated the important roles of Vit D_3 in regulating the fates of various cells, little is known about the effects of Vit D_3 on the physiological process of GCs and how it influences their characteristics.

The present study aimed to detect the VDR expression pattern throughout goat follicular development and to investigate the role of Vit D_3 in GCs proliferation and steroidogenesis. Elucidation of the effect of Vit D_3 on GCs performance would contribute to our understanding of its function in follicular development. Particularly, the goat industry would benefit from this research.

2. Materials and methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University (approval number: SYXK2011-0036) and were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Except for some specific reagents, all chemical and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and the media were obtained from Life Technologies (Carlsbad, CA, USA). All antibodies were obtained from commercial sources (Table 1). All experiments were carried out three times, separately.

2.1. Animals and sample collection

Ovaries of sexually mature ewes (Yangtze River Delta White Goat) were collected from a local abattoir (Taizhou, Jiangsu; 32° 00′ N, 119° 57′ E) during the breeding season (October to March). Ovaries were immediately immersed in Dulbecco's PBS (DPBS; Ca^{2+}- and Mg^{2+}-free; 30–35 °C) supplemented with 100 IU/ml penicillin and 50 mg/ml streptomycin, and transported to the laboratory within 2 h. Connective tissues and attached oviducts were removed after washing five times with DPBS. Six ovaries were randomly selected and fix in 4% formaldehyde for 24 h, then embedded in paraffin for immunohistochemistry. All visible antral follicles were dissected from remaining ovaries, measured with a caliper, and classified into 3 size classes (≤2 mm, 2–5 mm, and ≥5 mm). GCs were harvested from follicles of different size for analysis of VDR mRNA and protein expression. GCs from the remaining 2–5 mm follicles were harvested for *in vitro* culture.

2.2. Experimental design for in vitro culture of GCs

In the first experiment, the effects of Vit D_3 and FSH on GCs proliferation and steroidogenesis were evaluated. GCs were cultured as previously described [26], with minor modifications. Briefly, GCs (5×10^5 cells/well) were plated in 6-well plates in basic culture medium (BCM: DMEM/F12 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin) for 24 h. The medium was replaced with fresh BCM with different concentrations of Vit D_3 (Selleck, Houston, TX, USA) and FSH (Invitrogen, Carlsbad, CA, USA), and the cells were further incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 48 h. This study was performed based on a 2 × 2 factorial arrangements of Vit D_3 (0 and 10 nM) and FSH (0 and 10 ng/ml). After 48 h, the conditioned medium from each well was collected, and concentrations of E_2 and P_4 were measured using an ELISA kit (Blue Gene, Shanghai, china). The sensitivity of the assay was 1.4 pg/ml. The intra-assay coefficient of variation (CV) was 6.6%, and the interassay CV was 9.8%. Attached GCs were harvested using 0.25% EDTA-trypsin at 37 °C for 3 min followed by centrifugation at 2000 rpm for 5 min. The GCs were used for measuring cAMP with an ELISA kit (Blue Gene, Shanghai, China) following the manufacturer's protocol, and for a cell-cycle distribution assay.

The second experiment was aimed at investigating the precise signaling mechanism by which Vit D_3 influences GCs proliferation and steroidogenesis. *In vitro* culture was conducted similar to that in the first experiment, but with (or without) Vit D_3 (10 nM) treatment alone. After 48 h, attached GCs were collected for ROS assay and the remaining GCs were stored at −80 °C and for qRT-PCR and western blot analyses for mRNA and protein expression levels, respectively.

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted from GCs using Trizol reagent (Invitrogen) following the manufacturer's protocol. RNA concentration was determined on an ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Five micrograms of RNA in a final volume of 100 μl was transcribed into cDNA using a reverse transcription reagent kit (Takara, Dalian, China). The cDNA was stored at −20 °C until use.

2.4. Cloning of goat VDR cDNA

To investigate VDR expression in the GCs from 2–5-mm follicles and to obtain the coding sequence, two pairs of goat *VDR*-specific primers were designed using Primer 5.0 software (Table 2). The gene was amplified from cDNA using the following thermal program: 98 °C for 5 min, 35 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 90 s, and 72 °C for 7 min. Amplification products were separated on a 1.2% agarose gel. Purified PCR products of the expected size were cloned into the pMD19-T vector (Takara, Dalian, China) and transformed into *Escherichia coli* DH5a cells. Positive clones were randomly selected and sequenced by Tsingke Biological Technology (Beijing, China).

2.5. qRT-PCR analysis

qRT-PCR was carried out on an ABI 7500 Real-Time PCR System (Applied BioSystems, Carlsbad, CA, USA). Reactions were carried

Table 1
Information on the antibodies used in the study.

Antibodies	Cat no.	Company	Source	Specificity	Dilution of IHC	Dilution of WB
VDR	14526-1-AP	ProteinTech, Chicago, USA	Rabbit	Human, mouse, rat	1:200	1:500
FSHR	22665-1-AP	ProteinTech, Chicago, USA	Rabbit	Human, mouse	1:100	1:500
SOD2	NB100-1992	Novus Biologicals, Littleton, USA	Rabbit	Sheep and others	–	1:2000
CAT	21260-1-AP	ProteinTech, Chicago, USA	Rabbit	Sheep and others	–	1:1000
β-actin	bs-0061R	Bioss, Beijing, China	Rabbit	Sheep and others	–	1:2000

Table 2
Primer sequences used for this study.

Genes	Primer sequence (5′-3′)	Genebank No.	Species	Size (bp)	Target
VDR-cDNA	F: CCACCTTGGGACAGAGTCCT R: CAGGTCCAGGGTCACAGAAG	XM_018047873.1	*Capra hircus*	755	*VDR* gene full-length cDNA amplification
	F: CGTCCTCCTCCTGTTCGGAT R: GAACCCCAGCAGGTGTGGAAA	XM_018047873.1	*Capra hircus*	848	
VDR	F: TCCTCTCCAGACACAACGGA R: ACAGGTCCAGGGTCACAGAA	XM_018047873.1	*Capra hircus*	91	qRT-PCR
AMH	F: GTGGTGCTGCTGCTAAAGATG R: TCGGACAGGCTGATGAGGAG	XM_018050766.1	*Capra hircus*	104	qRT-PCR
AMHR	F: GTGCTTCTCCCAGGTCATAC R: AATGTGGTCATGCTGTAGGC	XM_005679901.2	*Capra hircus*	163	qRT-PCR
FSHR	F: CAAAGATCCTCCTGGTCCTGTTC R: GTTCCTGGTGAAGATGGCGTAG	NM_001285636.1	*Capra hircus*	77	qRT-PCR
CYP19A1	F: TCGTCCTGGTCACCCTTCTG R: CGGTCTCTGGTCTCGTCTGG	XM_013967046.2	*Capra hircus*	115	qRT-PCR
STAR	F: ACACCATGTGGAATGTCAGGCT R: CACACCTTTCAACAAGCAACCC	XM_013975437.2	*Capra hircus*	258	qRT-PCR
3β-HSD	F: CTATGTTGGCAATGTGGC R: ATCTCGCTGAGCTTTCTTAT	XM_013962473.2	*Capra hircus*	340	qRT-PCR
P21	F: CTAAGTGGGCAAATATGGGTCTGG R: CAGGATGCTACAGGAGCTGGAAG	XM_018039118.1	*Capra hircus*	107	qRT-PCR
P27	F: AAACCCAGAGGACACGCATT R: GGCAGGTCGCTTCCTTATCC	XM_005680816.3	*Capra hircus*	100	qRT-PCR
CDK1	F: GAATTAGCAACGAAGAAACC R: GTACAATTATCTGCTCTTGAC	NM_174016	*Bos taurus*	195	qRT-PCR
CDK4	F: CGTTGGCTGTATCTTTGC R: GATTCGCTTGTGTGGGTT	NM_001037594.2	*Bos taurus*	256	qRT-PCR
CyclinB1	F: GAGCCATCCTCATTGACTGGC R: CTTAGATGCTCTCCGAAGG	NM_001045872.1	*Bos taurus*	120	qRT-PCR
CyclinD1	F: CCGTCCATGCGGAAGATC R: CAGGAAGCGGTCCAGGTAG	XM_018043271.1	*Capra hircus*	108	qRT-PCR
SOD2	F: GTGAACAACCTCAACGTCGC R: GCGTCCCTGCTCCTTATTGA	XM_018053428.1	*Capra hircus*	300	qRT-PCR
GPx	F: ACATTGAAACCCTGCTGTCC R: TCATGAGGAGCTGTGGTCTG	XM_005695962.2	*Capra hircus*	216	qRT-PCR
CAT	F: CACTCAGGTGCGGGATTTCT R: ATGCGGGAGCCATATTCAGG	GQ204786.1	*Capra hircus*	159	qRT-PCR
GAPDH	F: CGACTTCAACAGCGACACTCAC R: CCCTGTTGCTGTAGCCGAATTC	NM_001034034.1	*Bos taurus*	119	qRT-PCR

out using FastStart SYBR Green Master mix (Roche, Mannheim, Germany), according to the manufacturer's protocol. qRT-PCR primers were designed using Primer 5.0 software (Table 2). Glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*) was used for normalization [17]. R^2 values for all standard curves generated ranged between 0.997 and 0.999, and the efficiencies of PCR were 90–110%. The relative mRNA levels of the target genes were expressed as $2^{-\Delta\Delta CT}$, and $\triangle$CT was calculated by subtracting CT(*GAPDH*) from CT (target gene).

2.6. Immunohistochemistry and immunocytochemistry

Immunohistochemistry was performed using our previously described method [27]. Rabbit anti-VDR was used as a primary antibody, and goat anti-rabbit IgG was used as a secondary antibody (#AP132P; Millipore, Billerica, MA, USA).

Immunocytochemistry was performed according to previously described method [28]. GCs (1×10^4 cells/well) were seeded onto cover slips of 24-well plates and were cultured at 37 °C in a humidified atmosphere with 5% for 48 h. The primary antibodies used were rabbit anti-FSHR and rabbit anti-VDR, while goat anti-rabbit IgG was used as a secondary antibody (#AP132P; Millipore). Negative controls were treated with Tris-buffered saline (TBS) instead of primary antibody. Sections were stained with DAB. Cells from each group were counted in 5 randomly selected fields using the Image-Pro Plus software (version 6.0 for Windows) under a light microscope (Nikon, Japan), at a magnification of 200 × . The number of positive cells was averaged for statistical analysis.

2.7. MTT analysis

Cell proliferation was determined using an MTT assay kit (Boster Co. Ltd., Wuhan, China) according to the manufacturer's instructions. Briefly, 3×10^3 cells/well were seeded in 100 μl of fresh BCM in 96-well plates for 24 h. *In vitro* culture was conducted as described under section 2.2. After 48-h culture, 100 μl of the supernatant of each well was added to 10 μl of MTT reagent and incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 4 h. The medium was removed and 100 μl of formazan solution was added to dissolve the formazan crystals, which are the chromogenic products of the reduction of MTT. The absorbance at 570 nm was measured using an ELISA reader (BD Biosciences, Franklin Lakes, NJ, USA). Cell proliferation was expressed as the fraction of surviving cells relative to untreated groups.

2.8. Flow-cytometric analysis of cell cycle and ROS production

Cell cycle and ROS production of GCs were analyzed by flow cytometry using propidium iodide (Invitrogen) and a ROS assay kit (Beyotime, Shanghai, China), respectively. For cell-cycle analysis, GCs were fixed with cold 70% ethanol overnight at −20 °C, washed with cold DPBS three times, incubated with 100 μl of RNaseA (10 ng/ml) for 30 min at 37 °C, and stained with 1 ml propidium iodide for 30 min. Cells at different stages of the cell cycle were identified flow cytometry (BD Biosciences). ROS levels of GCs were detected using a previously described method [29], with some modifications. Briefly, GCs were digested in the presence of Vit D_3

with or without FSH for 48 h, washed, and resuspended in DPBS. The harvested GCs were incubated with 1 ml (10 μm/l) 2′,7′-dichlorodihydrofluorescein diacetate at 37 °C for 20 min and washed twice in DPBS. Then, the cells were subjected to flow cytometry using wavelengths of 488 nm/525 nm (excitation/emission). Data were collected from at least 1×10^4 cells for each sample.

2.9. Western blot analysis

Total protein (of GCs of different-sized follicles and of GCs treatment with/without Vit D_3) was extracted with Cell Protein Extraction Reagent (Beyotime Biotechnology, Haimen, China) containing phenylmethanesulfonyl fluoride. Protein concentrations were determined with a BCA protein assay kit (Beyotime, Shanghai, China), and 40–60 μg of protein was separated on a 12% SDS-polyacrylamide gel and then transferred onto a polyvinylidene fluoride membrane (Millipore). Subsequently, membranes were blocked with 5% (w/v) fat-free milk for 2 h at room temperature, and then incubated at 4 °C overnight with primary antibodies against VDR, FSHR, SOD2, CAT and β-actin. After washing with TBS containing 0.05% Tween-20, the membranes were incubated with peroxidase-conjugated secondary antibody at room temperature for 1 h. The proteins were visualized with an enhanced chemiluminescence detection system (Fujifilm, Tokyo, Japan), and the chemiluminescence intensity of each protein band was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.10. Statistical analysis

All experiments were performed in triplicate, and data are expressed as the mean ± standard error of the mean (SEM). The distribution of all data was first confirmed for their agreement with normal distribution using the Kolmogorov-Smirnov goodness-of-fit test. Group means were compared using ANOVA followed by Tukey's test. All analyses were done with SPSS statistics (version 19.0 for windows). *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Goat VDR cloning and sequence analysis

A 1484-bp VDR cDNA fragment was obtained by splicing the two fragments from the GCs of goat (Fig. 1 and Fig. S1). The sequence has been submitted to GenBank (accession number KY307887.1). The full-length coding sequence is 1278 bp long and encodes 425 amino-acid residues (Fig. S1). To understand the evolutionary relationship with other species, a neighbor-joining tree was constructed using MEGA3.1 (Fig. 1B). The goat VDR is positioned with that of sheep in one clade, indicating that these are closest homologs.

3.2. VDR is expressed in the GCs of goat ovaries

As shown in Fig. 2, VDR was prominently localized to the GCs of follicles of all sizes analyzed (Fig. 2A–H). VDR signal was detected in oocytes from follicles of the primordial stage onward (Fig. 2A and B) and in the oocyte cytoplasm and GCs of primary (Fig. 2C) and secondary follicles (Fig. 2D). In antral stages, VDR was present in GCs and theca cells (Fig. 2E). VDR was not detected when sections were incubated with normal rabbit serum (Fig. 2F).

The mRNA and protein expression levels of VDR in goat GCs from follicles of different sizes are shown in Fig. 2. VDR protein expression significantly increased with increasing follicle diameter ($P < 0.05$, Fig. 2H and I). A similar trend was observed for mRNA expression (Fig. 2G); however, there was no significant difference between follicles of 2–5 mm and those ≥5 mm ($P > 0.05$).

3.3. Vit D_3 and FSH enhance cell proliferation of goat GCs cultured in vitro

After treatment of GCs with each of Vit D_3 and FSH or the combination thereof, the expression of FSHR (Fig. 3A–D) and VDR (Fig. 3E–H) was determined in all groups. In the groups treated with Vit D_3 and FSH alone, protein expression of FSHR and VDR were significantly increased as compared to that in the control group (Fig. 3J and K; $P < 0.05$). Moreover, Vit D_3 significantly

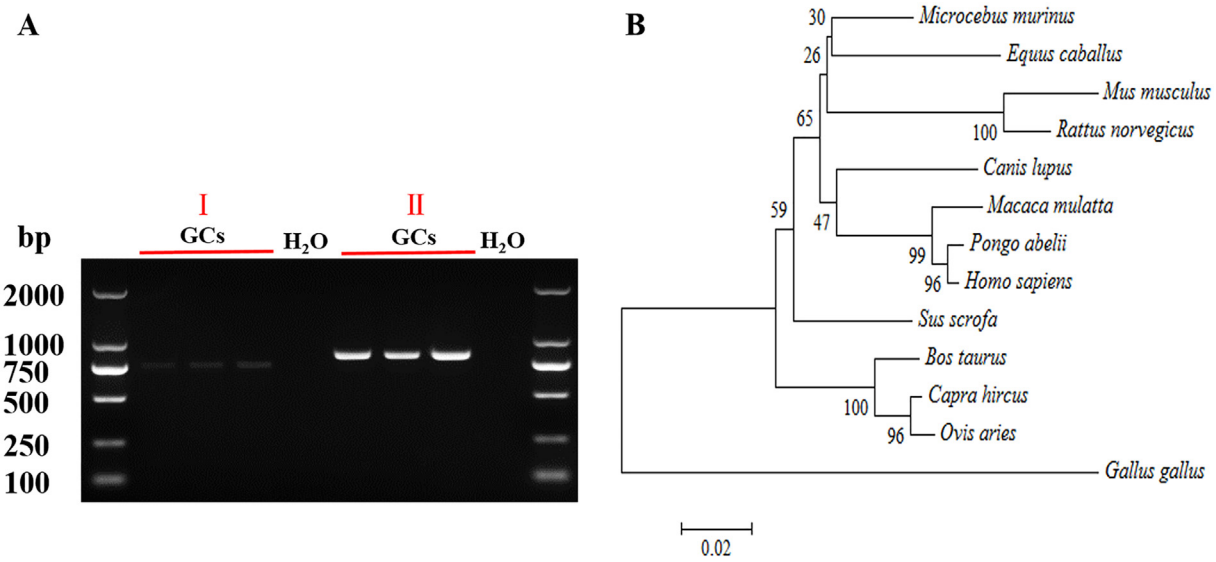

Fig. 1. Primers used for the isolation of the goat *VDR* gene. (A) Two pairs of specific primers designed for amplifying the open reading frame of VDR. (B) Phylogenetic tree generated from the amino-acid alignment of VDR sequences of 13 different species.

enhanced the positive effect of FSH on the protein expression of FSHR and VDR ($P < 0.05$).

To assess the effects of Vit D_3 and FSH on GCs proliferation, MTT assays were conducted (Fig. 4A). Vit D_3 and FSH alone progressively stimulated the proliferation of GCs from 2 to 5 mm follicles ($P < 0.05$). However, Vit D_3 did not exert a stimulatory effect on GCs proliferation in the presence of FSH ($P > 0.05$).

3.4. Effects of Vit D_3 and FSH on the cell cycle of goat GCs cultured in vitro

The cell cycle progressions were shown the accompanying proliferation effects, which the percentage of G0/G1 fraction was decreased and S fraction increased by treatment GCs with Vit D_3 or FSH alone respectively, when compared with control (Fig. 4B and C; $P < 0.05$). Vit D_3 did not exert positive effect on G2/M phase neither alone nor stimulating with FSH ($P > 0.05$).

To investigate the effect of Vit D_3 on GCs proliferation further, the expression of cell cycle-related genes (*P21*, *P27*, *CDK1*, *CyclinB1*, *CDK4* and *CyclinD1*) was analyzed by qRT-PCR (Fig. 5). Treatment with Vit D_3 alone significantly increased *CDK4* and *CyclinD1* mRNA expression, but it caused a significant decrease in *P21* mRNA expression ($P < 0.05$). No effects on *P27*, *CDK1* and *CyclinB1* mRNA expression were observed ($P > 0.05$).

3.5. Vit D_3 lowers ROS and induces certain antioxidant-related genes in goat GCs cultured in vitro

As shown in Fig. 6, treatment with Vit D_3 alone significantly decreased the ROS content as compared to the control group (Fig. 6A; $P < 0.05$). Meanwhile, both mRNA and protein expression of SOD2 and CAT were significantly increased in the 10 nM Vit D_3 group (Fig. 6B; $P < 0.05$). However, Vit D_3 had no significant effect on *GPx* mRNA expression (Fig. 6B; $P > 0.05$).

3.6. Vit D_3 and FSH improve steroidogenesis and enhance the cAMP pool

To examine whether Vit D_3 or FSH affects steroidogenesis in GCs, E_2 and P_4 concentrations were examined (Fig. 7A and B). Either Vit D_3 or FSH alone significantly stimulated E_2 and P_4 production by GCs after 48 h ($P < 0.05$). Vit D_3 further potentiated the stimulatory effect of FSH on E_2 (Fig. 7A, $P < 0.05$), but not P_4 (Fig. 7B; $P > 0.05$) production. In addition, the involvement of cAMP as a second messenger system was examined by determining cAMP levels (Fig. 7C). A trend similar to that of E_2 was observed for cAMP; Vit D_3

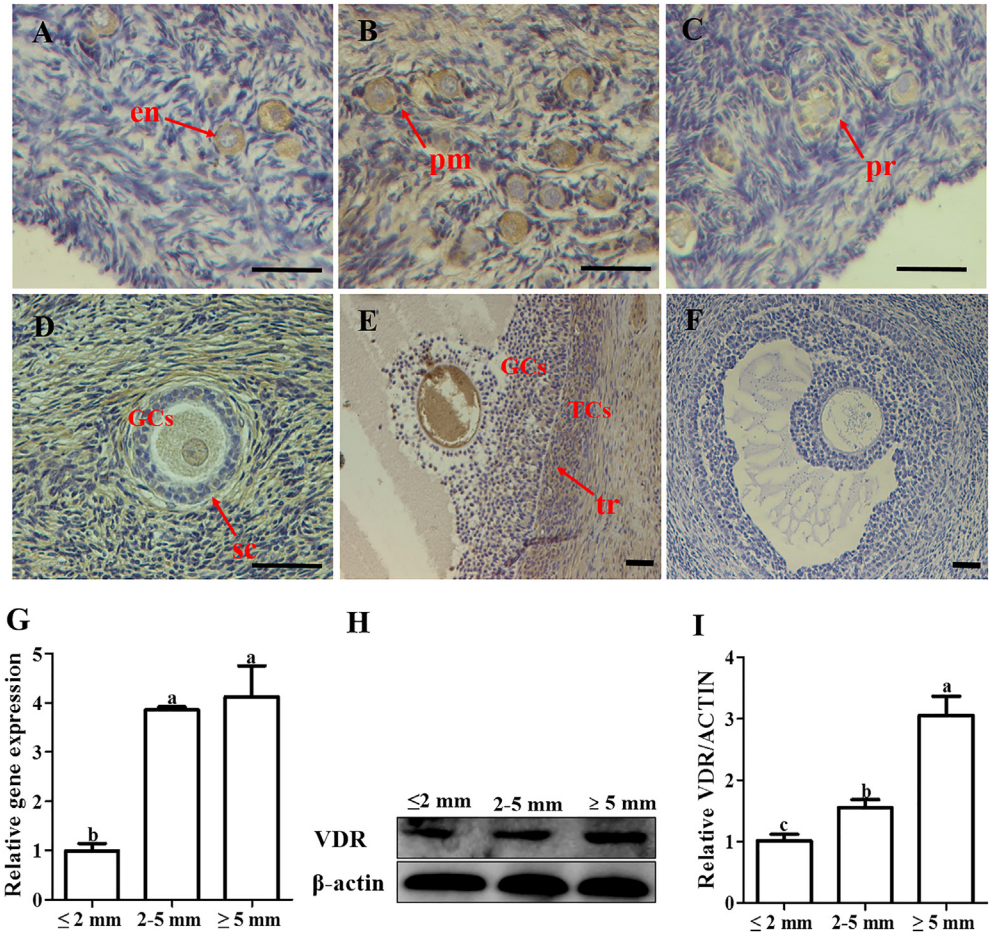

Fig. 2. VDR is expressed in the GCs of goat follicles. Immunohistochemical localization of VDR in goat ovaries (A–F). qRT-PCR (G) and western blot (H and I) analyses were performed to detect VDR expression in GCs from follicles of different sizes (≤2 mm, 2–5 mm, and ≥5 mm). Data for each group were obtained from three animals with three technical replicates per animal, and are expressed as the mean ± SEM. Different superscript letters (a–c) indicate statistically significant differences ($P < 0.05$). en: egg nest; pm: primordial follicle; pr: primary follicle; sc: secondary follicle; tr: tertiary follicle; GCs: granulosa cells; TCs: theca cells. A–D (400 ×); E, F (200 ×). Scale bars correspond to 50 μm.

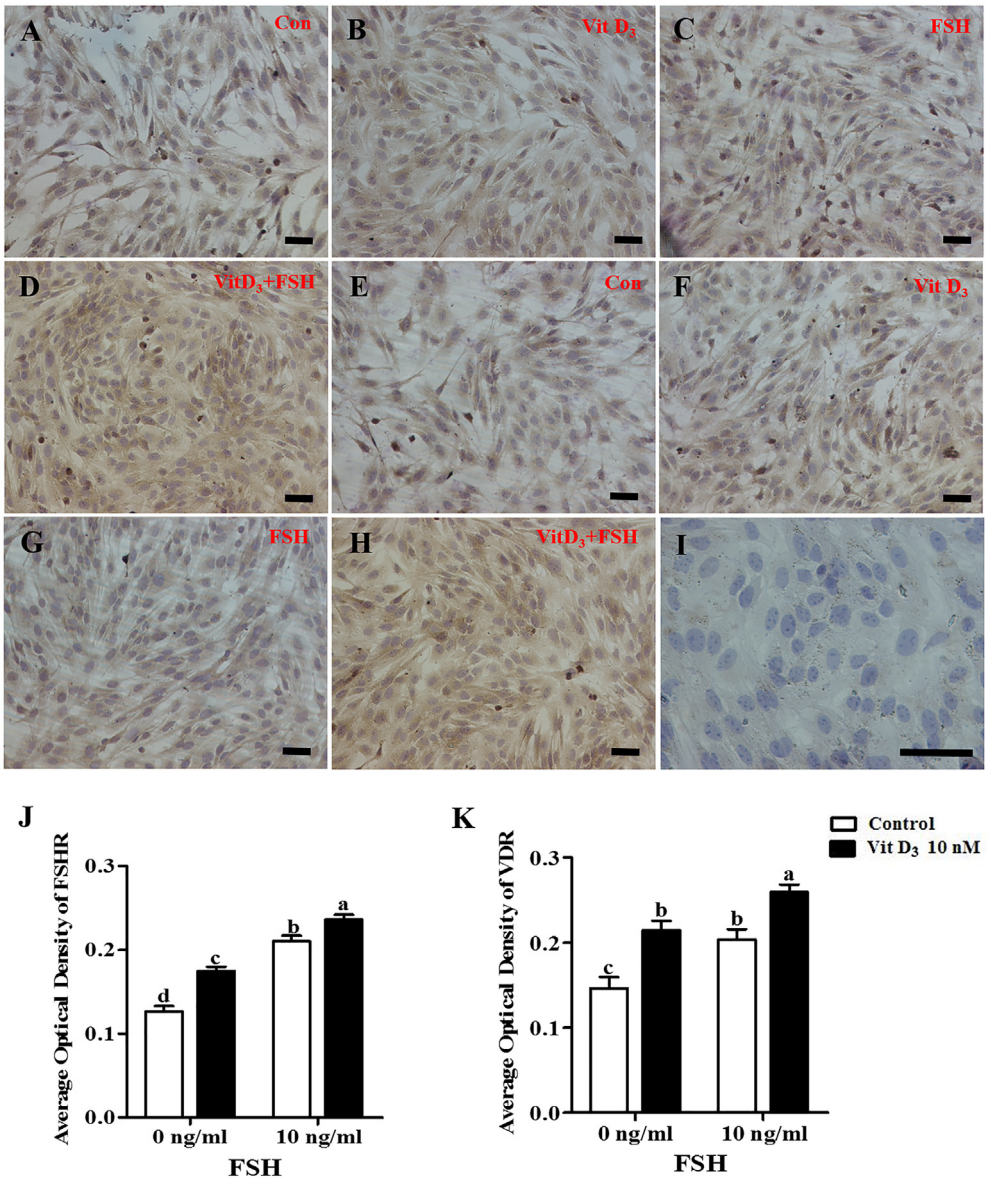

Fig. 3. Vit D_3 and FSH stimulate goat GCs proliferation. Immunocytochemistry for FSHR (A–D) and VDR (E–H) in different treatment groups after 48-h culture. The different treatments include the control group (A, E), Vit D_3 group (B, F), FSH group (C, G), and Vit D_3 with FSH group (D, H). Average optical densities of FSHR (J) and VDR (K) expression in different treatment groups are shown. Negative control (I). A–H (× 200), I (× 400). GCs: granulosa cells. Scale bars correspond to 50 μm.

and FSH each had a significant positive effect on the cAMP concentration in the cells, which was enhanced when both were combined ($P < 0.05$).

3.7. *Vit D_3 affects specific steroidogenic enzymes in goat GCs cultured in vitro*

The result of qRT-PCR is shown in Fig. 8, treatment with 10 nM Vit D_3 in the absence of FSH had no effect on *AMH* and *CYP19A1* expression ($P > 0.05$), while it significantly decreased in the *AMHR* and *FSHR* mRNA levels ($P < 0.05$) and increased those of *VDR*, *StAR* and *3β-HSD* (Fig. 8 A; $P < 0.05$). Protein expression of FSHR was significantly decreased upon treatment with Vit D_3 (Fig. 8 B; $P < 0.05$). Although the protein expression of VDR in the Vit D_3 group was slightly higher than that in the control group, the difference was not significant (Fig. 8 B; $P > 0.05$).

4. Discussion

Increasing evidences indicates that Vit D_3 might play an important role in reproduction in both males and females and exerts its effects through VDR [2]. In accordance herewith, in the current study, VDR expression was detected in GCs from goat follicles, which is supported by previous studies that reported the VDR expression in mouse [3,5] and rat [6] ovarian follicles. Furthermore, we found that VDR expression in the GCs increased with increasing follicle size, which is corroborated by similar findings in a previous study [30]. Together, these results suggest that Vit D_3 might play an important role during follicular development.

Previous research has indicated that an appropriate amount of Vit D_3 can affect follicle development indirectly by promoting GCs proliferation [30]. To understand how Vit D_3 influences follicle development, we investigated the effects of Vit D_3 with or without FSH on proliferation of goat GCs *in vitro*. Treatment with either

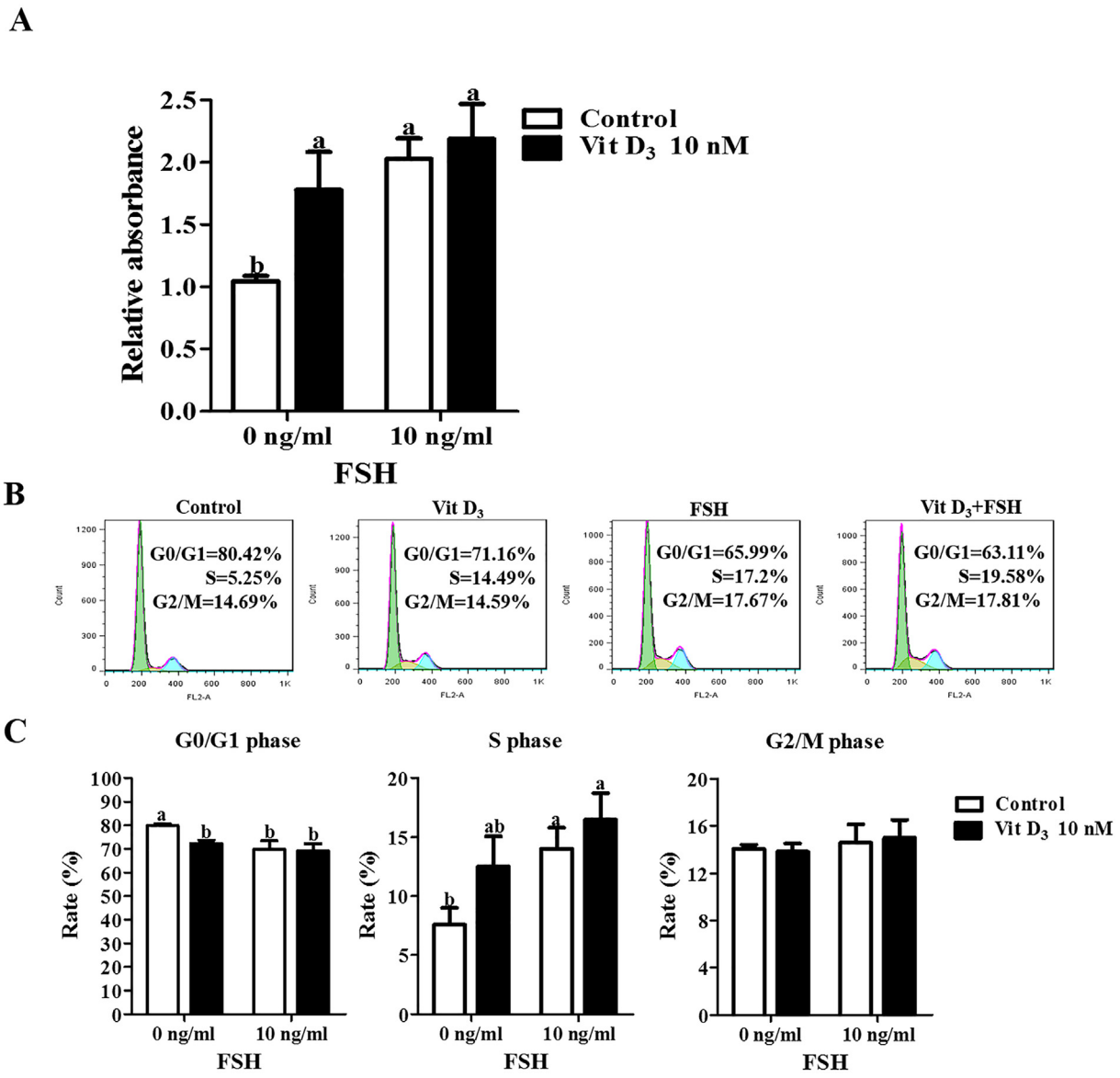

Fig. 4. Vit D_3 and FSH stimulate goat GCs cell-cycle progression. Effect of Vit D_3 and FSH on cell proliferation (A) and cell-cycle progression (B, C) in different treatment groups of GCs after 48 h of culture. (A) Absorbance of MTT at 570 nm was used to detect proliferation by ELISA. (B) Cell-cycle distribution was analyzed by flow cytometry. (C) Histograms represent the percentage of GCs in different phases of the cell cycle. Data for each group were from three animals with three technical replicates per animal, and expressed as the mean ± SEM. Different superscript letters (a, b) indicate statistically significant differences ($P < 0.05$).

10 nM Vit D_3 or 10 ng/ml FSH alone or the combination thereof promoted GCs proliferation, which was corroborated by the results of immunocytochemistry. Previous reports have shown that 10 nM Vit D_3 has an inhibitory effect on cancer cell proliferation [22,31,32], which may be owing to cell-type-dependent differences in the requirement for Vit D_3.

However, the mechanism of Vit D_3 in regulating GCs proliferation is still not fully understood. Cell proliferation and apoptosis are closely related to the cellular oxidative stress, whereas the overproduction of ROS can destroy the cellular structure and thus inhibit cell proliferation [33,34]. Our data showed that ROS production was dramatically (>40%) decreased upon addition of 10 nM Vit D_3 alone. At low concentrations, ROS serve as a key signal molecules in proliferation [35]. Therefore, our data suggest that Vit D_3 can induce GCs proliferation through a mechanism that is initiated by decreasing the ROS level. When cells are exposed to oxidative stress, ample antioxidant enzymes will be produced to counter the damaging effects of ROS, and a linear relationship between oxidative stress and the mRNA expression of antioxidant-related genes has been reported [26]. Accordingly, antioxidant-related enzymes (SOD2 and CAT) were found to be upregulated in GCs treated with Vit D_3. We observed no effect of Vit D_3 on *GPx* mRNA expression, which indicates that Vit D_3 specifically affects SOD and CAT mRNA and protein expression. This result was consistent with previous studies [23,36]. Therefore, we speculate that Vit D_3 might decrease the ROS level by improving SOD2 and CAT activities.

Cell-cycle control represents a major regulatory mechanism of cell growth, which is regulated by several types of cyclin, cyclin-dependent kinase (CDK), and cyclin-dependent kinase inhibitor (CKI) [37]. In the present study, treatment of GCs with Vit D_3 or FSH alone induced cell cycle arrest from G0/G1 to S phase, which was accompanied by the upregulation of *CDK4* and *CyclinD1* and the downregulation of *P21* expression. CDK function is tightly regulated

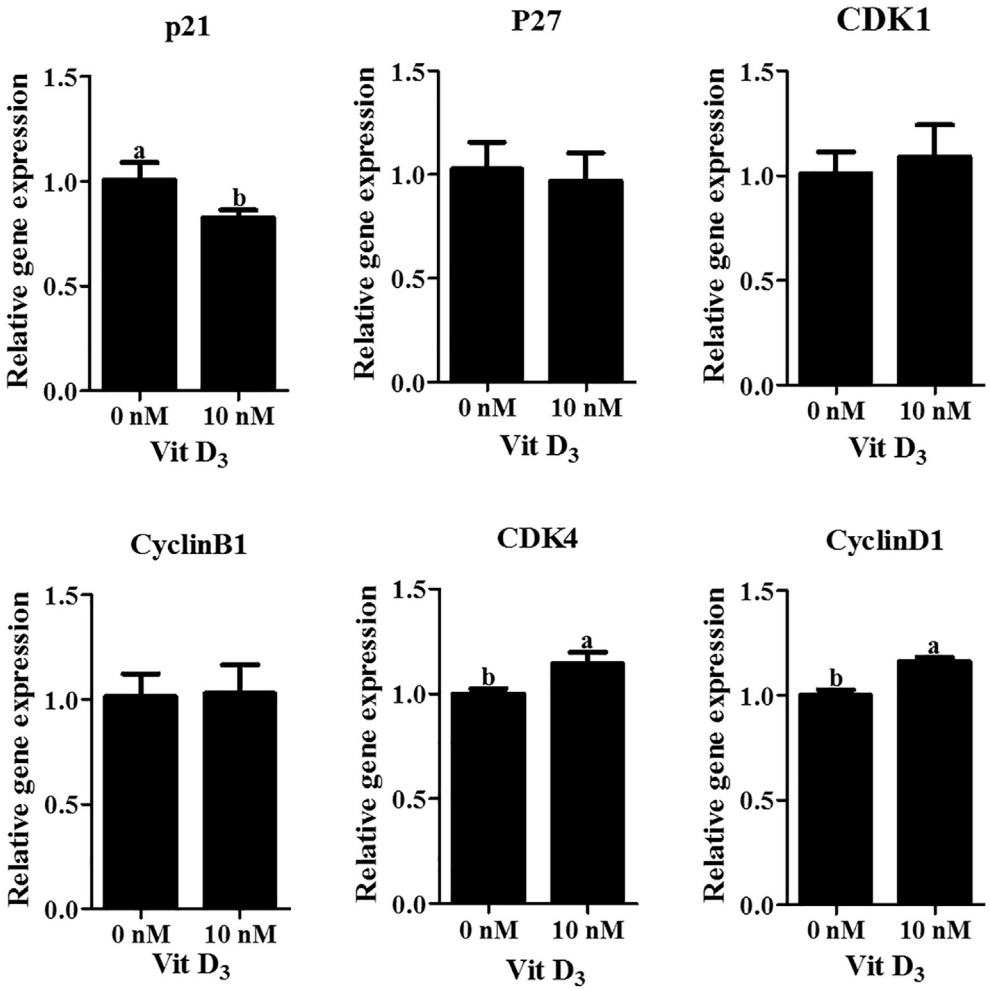

Fig. 5. Vit D_3 alters the mRNA expression of cell cycle-related genes in GCs. The effects of Vit D_3 and FSH on cell-cycle-regulatory genes (*P21*, *P27*, *CDK1*, *CyclinB1*, *CyclinD1*, and *CDK4*) in different treatment groups of GCs were determined after 48 h of culture. Data for each group were from three animals with three replicates per animal, and are expressed as the mean ± SEM. Different superscript letters (a, b) indicate statistically significant differences ($P < 0.05$).

by CKIs, such as *P21* and *P27* [37,38]. Activation of CyclinD1−CDK4 complexes is required for cells to pass the G1/S restriction point [39]. Our present findings are consistent with recent evidence suggesting that Vit D_3 induces G1/S phase arrest via the upregulation of *CDK4* and *CyclinD1* and inhibition of *P21* expression, thereby inducing the activation of pRb, which results in the release of E_2F transcription factors [40,41].

The finding that VDR was present in goat GCs suggested that it may be related to steroid hormone synthesis and production. Indeed, either Vit D_3 or FSH alone significantly stimulated E_2 and P_4 production. Other studies have also demonstrated that Vit D_3 increases E_2 and P_4 release in human and porcine GCs [17,18,42]. Moreover, our data showed that Vit D_3 further potentiated the stimulatory effect of FSH on E_2 production. This is in contrast to findings of Smolikova et al. [18] and Merhi et al. [17], which indicated that Vit D_3 does not affect FSH-induced E_2 production in human and porcine. This discrepancy may be explained by the different animal species investigated and dose-response effects. P_4 synthesis is a complex process modulated by aromatases including StAR and 3β-HSD [43]. The higher mRNA expression of *3β-HSD* and *StAR* upon the addition of Vit D_3 observed in the current study suggests that Vit D_3 can enhance the expression of aromatase-related genes, consequently promoting P_4 synthesis by GCs. This is corroborated by previous reports [17,44] that Vit D_3 supplementation can improve P_4 concentration by enhancing 3β-HSD activity in human and porcine GCs. Moreover, GCs exposed to high concentrations of FSH *in vitro* or *in vivo* are readily highly luteinized and ultimately form a corpus luteum that produces large amounts of P_4 [17]. Thus, our findings suggest that Vit D_3 may facilitate GCs luteinization by increasing their P_4 production, which improves the endometrial environment for pregnancy.

CYP19A1 aromatase is known to be critical for E_2 synthesis [45]. However, under our experimental conditions, cells treated with Vit D_3 but without FSH remarkably increased their E_2 production but not E_2 mRNA expression. This phenomenon could be the effect of Vit D_3 on aromatase activity, which has been shown to be dose-dependent and tissue-specific [17,46]. Some previous studies reported that cAMP/PKA act as mediators associated with Vit D_3 [47,48]. Based on these findings, we speculated that the addition of Vit D_3 would affect E_2 production via influencing the intracellular cAMP level. This hypothesis was verified by our observation that Vit D_3 alone as well as in combination with FSH increased the GCs cAMP content.

AMH, upon interacting with its highly specific receptor AMHR, inhibits primordial to primary follicle transition and decreases FSH sensitivity, which regulates follicle selection during the estrous

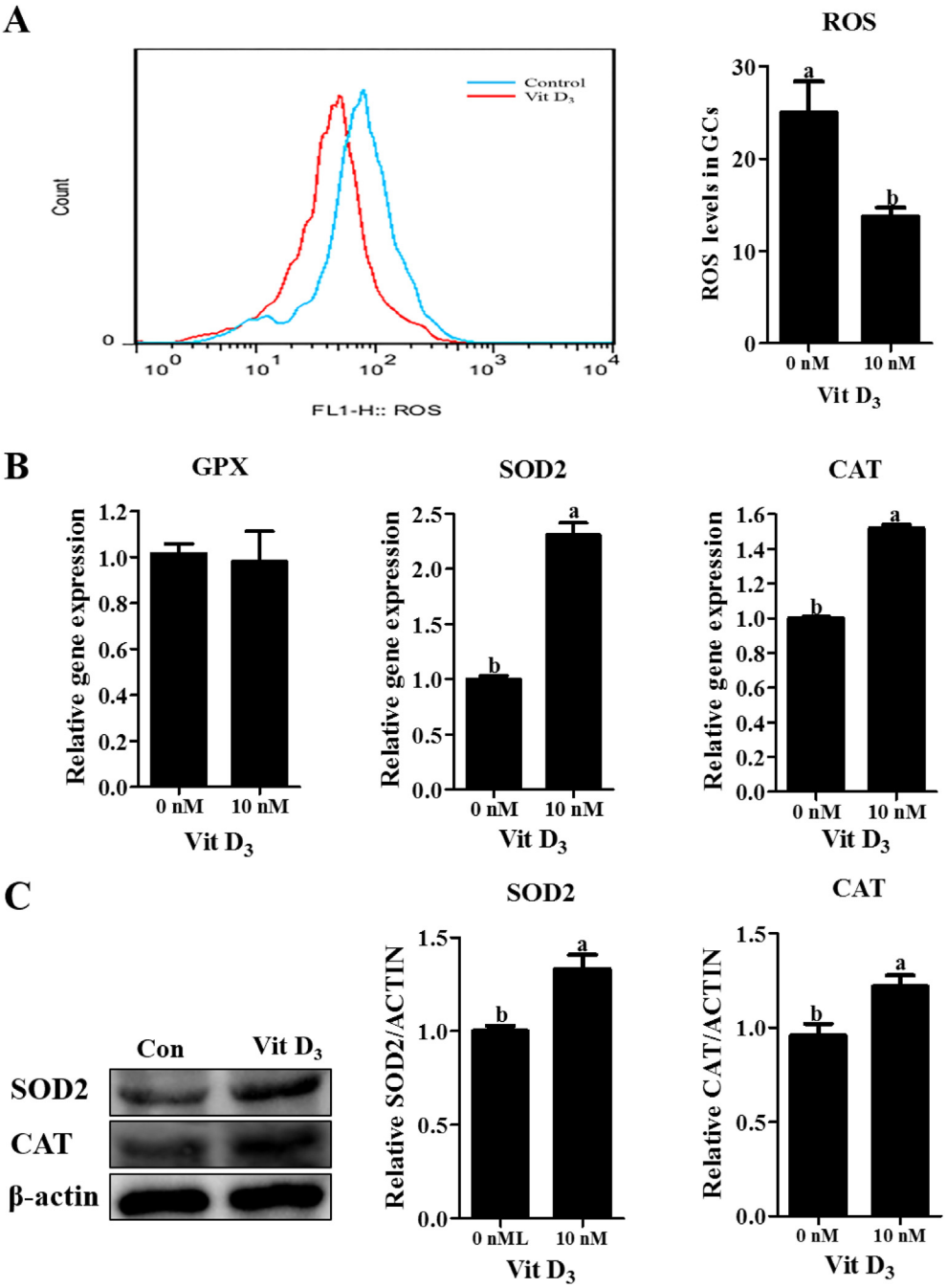

Fig. 6. Vit D_3 inhibits intracellular ROS levels and increases antioxidant-related gene expression. Intracellular ROS generation was quantified by flow cytometry (A). For each sample, 1×10^4 cells in the gated region were analyzed. mRNA and protein expression of antioxidant-related genes (*GPX*, *SOD2*, and *CAT*) in different groups (B). Data for each group were from three animals with three technical replicates per animal, and are expressed as the mean ± SEM. Different superscript letters (a, b) indicate statistically significant differences ($P < 0.05$).

cycle [49,50]. We found that Vit D_3 significantly decreased *AMHR* and *FSHR* mRNA expression, which could explain how Vit D_3 inhibited the effect of AMH on GCs proliferation via impeding *AMHR* expression. Similar patterns of *AMHR*, *FSHR* and *AMH* expression in human follicles have been reported [17]. FSHR expression reportedly peaks in GCs from small immature follicles and gradually reduces during folliculogenesis [51,52]. In the present study, Vit D_3 repressed *FSHR* mRNA and protein expression, suggesting that the GCs were in a more mature follicular state, which is corroborated by their size (2–5 mm) on the basis of which they were selected for analysis. Additionally, treatment with Vit D_3 alone increased VDR mRNA and protein expression. Which pathway is involved in the VDR increase by Vit D_3 remains unknown and requires further studies.

We used a luteinized GCs model to investigate the effect of Vit D_3 on goat GCs proliferation and steroidogenesis. Although this model may not be ideal for studying the physiological mechanism underlying proliferation and steroid hormone synthesis and secretion via Vit D_3 *in vivo*, our previous study [26] and others [17,18,53] have shown that *in vitro* culture of luteinized GCs can be

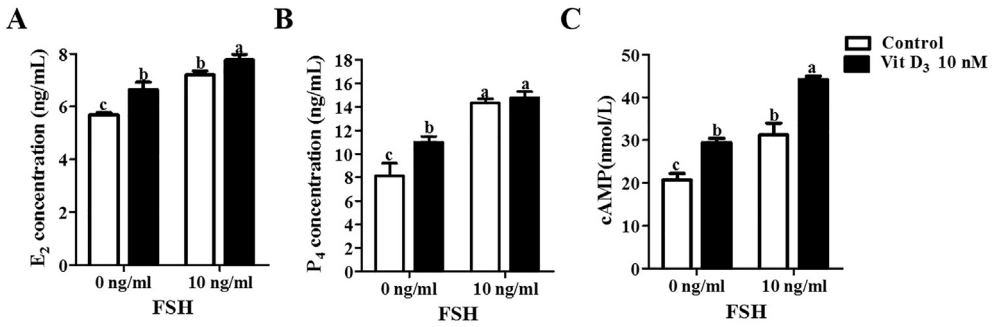

Fig. 7. Vit D_3 and FSH enhance steroidogenesis and cAMP content. Estradiol (A) and progesterone (B) production in the culture medium and cAMP level (C) in GCs of various treatment groups. Data for each group were from three animals with three technical replicates per animal, and are expressed as the mean ± SEM. Different superscript letters (a–c) indicate statistically significant differences ($P < 0.05$).

used to study GCs functions related to apoptosis and steroid hormone production.

In conclusion, the present study demonstrated that VDR is expressed in goat GCs and suggests that Vit D_3 plays an important role in the regulation of GCs proliferation during follicular development. Meanwhile, Vit D_3 can influence AMH signaling, steroidogenesis, and oxidative stress through regulating certain key factors during follicle selection (Fig. 9). However, there is evidence

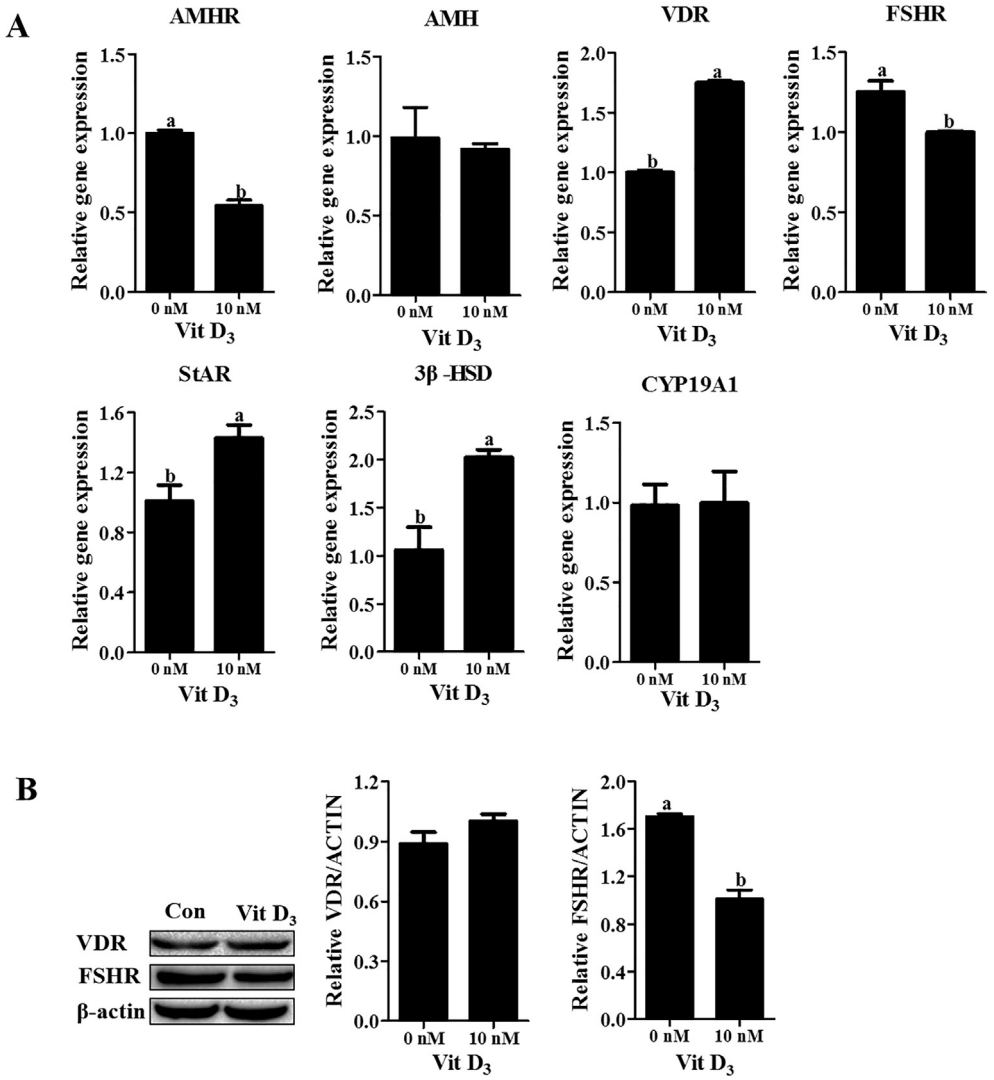

Fig. 8. Vit D_3 alters the mRNA expression of *AMHR*, *VDR*, *3β-HSD*, and *FSHR* in GCs. Data for each group were from three animals with three technical replicates per animal, and are expressed as the mean ± SEM. Different superscript letters (a, b) indicate statistically significant differences ($P < 0.05$).

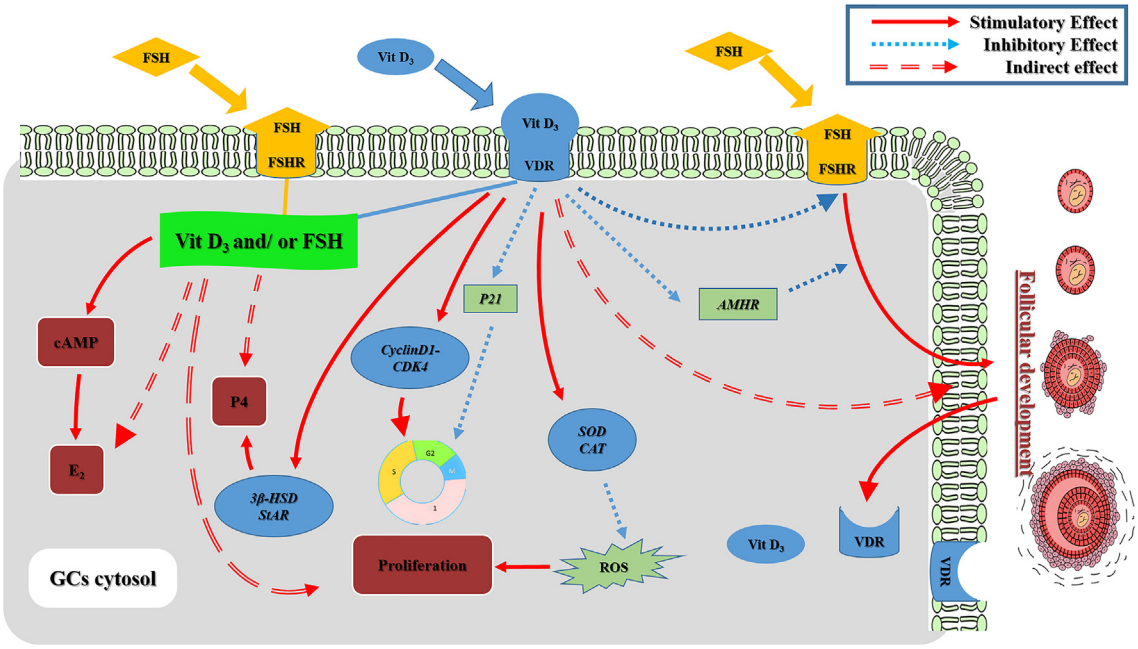

Fig. 9. Vit D_3 and FSH promote P_4 and E_2 production by increasing *3β-HSD* mRNA and cAMP levels. Vit D_3 induces G1/S phase arrest via the upregulation of *CDK4* and *CyclinD1* and inhibition of *P21* expression. Vit D_3 decreases the ROS level by promoting *SOD* and *CAT* mRNA expression. Additionally, Vit D_3 downregulates *FSHR* and *AMHR* mRNA levels.

indicating that steroid hormone production can be mediated by different pathways, such as cAMP/PKA, PI3K, WNT, and MAPK signaling pathways [54–57]. Therefore, further studies are needed to determine which pathways (direct or indirect) Vit D_3 employs to influence goat GCs functions.

Conflict of interest

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.theriogenology.2017.08.002.

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