CITED4 mediates proliferation, apoptosis and steroidogenesis of Hu sheep granulosa cells *in vitro*

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Abstract

Being a novel target of luteinizing hormone (LH), the effect of CREB-binding protein/P300-interacting trans-activator with ED-rich tail member 4 (*CITED4*) gene on the proliferation, apoptosis, and steroidogenesis of ovarian granulosa cells (GCs) in Hu sheep was investigated. The presence of *CITED4*, CREB-binding protein (CBP), CCAAT/enhancer-binding protein alpha (C/EBP α) and -beta (C/EBP β) proteins was demonstrated in GCs and luteal cells. *CITED4* protein in GCs was induced by LH, and *CITED4* overexpression moderately increased GC responses to LH. In contrast, *CITED4* knockdown in GCs decreased prostaglandin (PGE₂)-induced LH target gene levels. Moreover, PGE₂-stimulated *CITED4* mRNA expression was blocked by ERK1/2 inhibition (U0126), suggesting that *CITED4* is a downstream target of the ERK1/2 pathway in sheep GCs. In contrast to *CITED4* knockdown, *CITED4* overexpression promoted GC proliferation, inhibited apoptosis, upregulated cell cycle-related genes, and downregulated apoptosis-related genes. Additionally, *CITED4* overexpression induced cell cycle transition from S to G₂/M phase. No effect was observed with *CITED4* knockdown. *CITED4* overexpression increased progesterone (P₄) production levels and *STAR* mRNA expression, whereas *CITED4* knockdown decreased P₄ production and *STAR* and β -HSD mRNA expression levels. Thus, our results suggest that *CITED4* is involved in regulating the expression of LH-induced genes and the ERK1/2 pathway and the proliferation, apoptosis, and steroidogenesis in Hu sheep GCs by modulating the expression of related genes. These findings will help understand the role of *CITED4* in follicular development and ovulation of pre-ovulatory follicles.

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Introduction

In mammals, primary ovarian functions include mature oocyte production and steroids synthesis that support secondary sexual characteristics and pregnancy (Newman *et al.* 2008). Accumulating evidence indicates that ovarian granulosa cells (GCs) support follicle development and maturation by secreting steroids, growth factors, and cytokine factors (De Cesaro *et al.* 2018). GC proliferation is essential in the recruitment, selection, ovulation of follicles, and atresia (McGee & Hsueh 2000). GC apoptosis can impair folliculogenesis, resulting in follicular atresia (Matsuda *et al.* 2012). Therefore, elucidating GC function is important for understanding follicular development and female fertility.

Folliculogenesis and ovulation are tightly regulated processes in which sequential responses are triggered by pituitary gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) (Shimada & Yamashita 2011, Hackbart *et al.* 2017). GCs respond

rapidly to LH, which affects the expression of LH-induced genes that upregulate growth and transcription factors *in vivo*, to promote ovulation and luteinization (Carletti & Christenson 2009). Notably, LH stimulates the mural GCs of pre-ovulatory follicles to secrete EGF family proteins, such as amphiregulin (AREG) and epiregulin (EREG), which are intra-follicular mediators of LH activity (Park *et al.* 2004). Similarly, in rodent GCs, expression levels of prostaglandin-endoperoxide synthase 2 (PTGS2), tumor necrosis factor-inducible gene 6 (TNFAIP6), and pentraxin 3 (PTX3) increased significantly after human chorionic gonadotropin (hCG) injection (Espey & Richards 2002, Zhang *et al.* 2014). Recently, CREB-binding protein/P300-interacting transactivator with ED-rich tail member 4 (*CITED4*) was demonstrated as a novel LH-target for triggering ovulation through the ERK1/2 pathway. Transfection of *CITED4* regulated the expression of LH-target genes in mouse GCs (Zhang *et al.* 2014), suggesting that *CITED4* is required for stimulating LH-target gene expression during follicular development and ovulation. However, *CITED4* function

during follicular development and ovulation in ruminant GCs of Hu sheep has not been thoroughly investigated.

CITED4 may regulate gene transcription and function by binding to CREB-binding protein (CBP)/p300 as a co-activator (Yahata *et al.* 2002). Moreover, CITED4-CBP also binds to CCAAT/enhancer-binding protein beta (C/EBP β), thereby promoting its transcriptional activity (Lee *et al.* 2010, Zhang *et al.* 2014). CITED4, CBP, and C/EBP β are highly expressed in mouse (Zhang *et al.* 2014) or porcine (Zhen *et al.* 2014, Yuan *et al.* 2018) GCs from pre-ovulatory follicles. C/EBP β is an essential transcription factor for LH-induced ovulation and luteinization (Fan *et al.* 2011). C/EBP β also regulates porcine GC apoptosis and proliferation (Zhen *et al.* 2014, Yuan *et al.* 2018) and enhances estradiol (E₂) and progesterone (P₄) secretion (Zhen *et al.* 2014). Similar effects are observed in P₄ production by a human granulosa tumor cell line (Mizutani *et al.* 2014). These results support the hypothesis that C/EBP β is involved in folliculogenesis and ovulation. However, CITED4 functions in cell proliferation, apoptosis, and steroidogenesis in pre-ovulatory GCs remain unexamined.

To provide insights into the role of CITED4 in regulating follicular development in Hu sheep, the present study aimed to detect the expression of CITED4, CBP, C/EBP α , and C/EBP β in ovaries and investigate the role of CITED4 in GC proliferation, apoptosis, and steroidogenesis. Additionally, we investigated whether direct ERK1/2 inhibition could block CITED4 and LH-target gene expression in the GCs of Hu sheep.

Materials and methods

Ethics and reagents

All experimental procedures, including animal care, were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University. All reagents used in this study were purchased from Life Technologies, unless otherwise mentioned. All antibodies were purchased from commercial suppliers (Table 1).

Sample collection

During the breeding season (from October to January), Hu sheep ovaries were collected from a local abattoir (Taicang, Jiangsu; 121°10'E, 31°45'N), immediately immersed in sterilized physiological saline (supplemented with 100 IU/L penicillin and 50 mg/L streptomycin; at 30–35°C), and transported within 30 min to the laboratory. After washing five times with physiological saline, the connective tissues were removed from the collected ovaries. For immunohistochemical assay, ten ovaries were randomly selected, fixed in 4% formaldehyde for 24 h, and embedded in paraffin. Corpora lutea were dissected from ten ovaries, measured with a caliper, and classified into three size ranges (5–6, 7–8 and 9–10 mm diameter). Meanwhile, female reproductive organs (oviduct, ovary, uterine horn, hypothalamus, and pituitary) were collected from five ewes during estrus, at the Jiangyan Experimental Station (Taizhou, Jiangsu Province, China). The aforementioned samples were immediately frozen in liquid nitrogen and stored at –80°C.

GCs were isolated according to our previous protocol (Zhang *et al.* 2016). Briefly, follicular fluid was aspirated from healthy follicles (2–5 mm diameter). Subsequently, GCs were pelleted by centrifugation at 1000× *g* for 5 min and resuspended in red blood cell lysis buffer for 1 min. After washing with Dulbecco's PBS, the cell pellets were resuspended in 0.3% hyaluronidase for 90 s. Finally, the GCs were resuspended in culture medium (DMEM/nutrient mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin) at 37°C in a humidified atmosphere containing 5% CO₂. Notably, after 24–48 h of culture with serum, most GCs were converted into luteinized GCs (Tosca *et al.* 2010).

Cloning of CITED4 in Hu sheep

To obtain the coding DNA sequence region of the *CITED4* gene, specific primers with an amplicon of 989 bp were designed using the Primer 5.0 software and are listed in Table 2. RNA isolated from Hu sheep ovaries was transcribed into cDNA and amplified using RT-PCR based on our previous protocol (Yao *et al.* 2017a). Briefly, the following parameters were used: 98°C for 3 min; 35 cycles at 98°C for 10 s, 60°C for 30 s, and 72°C for 90 s or 45 s; and then a final extension

Table 1 Details of antibodies used.

Antibodies	Cat No.	Source	Dilution of IHC	Dilution of WB
CITED4	ab134072	Abcam, Cambridge, MA, USA	1:100	1:1000
CBP	AF0139	Affinity Biosciences, Cincinnati, USA	1:100	—
C/EBP α	A0904	ABclonal, Wuhan, China	1:100	—
C/EBP β	A0711	ABclonal, Wuhan, China	1:100	1:500
ERK1/2	AF1051	Beyotime, Shanghai, China	—	1:1000
p-ERK1/2	AF1891	Beyotime, Shanghai, China	—	1:1000
PCNA	Ab15497	Abcam, Cambridge, MA, USA	—	1:1000
BAX	50599-2-Ig	ProteinTech, Chicago, IL, USA	—	1:2000
β -actin	bs-0061R	Bioss, Beijing, China	—	1:2000
GAPDH	60004-1-Ig	ProteinTech, Chicago, IL, USA	—	1:2000
Goat anti-Rabbit IgG	SA00001-2	ProteinTech, Chicago, IL, USA	1:100	1:2000

Cat No., category number; IHC, immunohistochemistry; WB, western blot.

Table 2 Primer sequences used for this study.

Items	Primer sequence (5'–3')		Fragment Size (bp)	Genebank No.	Purpose
	Forward	Reverse			
CITED4	TTCTGTGTACTGGGCTGCG	GGAGTCCGAAAAGGGGGTTC	989	XM_004003498.4	RT-PCR
CITED4	ATGGACGCCGAATCATCG	CCCAAGTCCGAGAAGCAGTC	128	XM_004003498.4	qRT-PCR
AREG	CGCCATGACCTTCACAGCTA	CCTTCAGTTACGCTACGGCA	143	XM_012180164.3	qRT-PCR
EREG	TGGTGTCCGGTGTGAACATT	GGATCCCCTGAGGTCACTCT	195	XM_027971077.1	qRT-PCR
PTGS2	CCAGCCGCACTAGAGAAAGT	AATGGTGTCCGGCTTCTAC	266	NM_001009432.1	qRT-PCR
PTX3	TGGTCGCTGATGCTGTGATT	CCACCACCTACGCCATTCTT	193	XM_004003220.4	qRT-PCR
TNFAIP6	AGGGCAGAGTTGGATACCTT	ACCACCACACTCCTTTGCAT	146	XM_004004704.3	qRT-PCR
HAS2	GGAGACATATCGCTGCTGCT	GGCAAAACACTGTTAGGCGG	184	XM_004011666.3	qRT-PCR
Star	GGGCATCTCAAAGACCAG	TCCACCACCACCTCCAAC	120	NM_001009243.1	qRT-PCR
3β-HSD	ATCCACACCAGCACCATAG	TTCCAGCACAGCCTTCTC	144	NM_001135932.1	qRT-PCR
Caspase3	GGCTCTGAGTGTTTGGGGAA	CCTGGACAAAGTCCGTGGT	131	XM_027962551.1	qRT-PCR
Caspase8	TGAAGGTTCCAGGATTCCGC	GGCTTAGGAAGTTGAGGGCA	136	XM_012142484.3	qRT-PCR
BAX	CGAGTGGCGGCTGAAAT	GGTCTGCCATGTGGGTGTC	286	XM_027978594.1	qRT-PCR
Bcl-2	CGCATCGTGGCCTTCTTT	CGGTTCAGGTAATCGGTATC	113	XM_027960877.1	qRT-PCR
CyclinD1	ACATGGAGCTGGTCTGGTGA	GGAGGGTGGGTGGAAATGAA	188	XM_027959928.1	qRT-PCR
CDK4	CAGTGGCTGAGATTGGTGTG	ACCTCCCGAACGGTGCTGAT	148	XM_012158548.3	qRT-PCR
CDK1	ATGGCTGGATCTGCTCTCG	TGCTCTTGACACAACACAGGA	154	NM_001142508.1	qRT-PCR
CyclinB1	GCTTGGAGACATCGGTAACA	GGAGCCTTTTCCAGAGGTTTGT	129	XM_004016916.3	qRT-PCR
GAPDH	GTCAAGGCAGAGAACGGGAA	GGTTCACGCCCATCACAAAC	232	XM_012166462.1	qRT-PCR

at 72°C for 6 min. After gel electrophoresis, PCR products of the correct size were cloned into a pMD19-T vector (6013; Takara, Dalian, China). Cloned vectors were transformed into *Escherichia coli* DH5a cells (9057; Takara) using standard procedure. After sequencing, the amino acid sequence was predicted using BLAST and aligned with sequences from other species using the DNAMAN software. The sequence of CITED4 from Hu sheep was submitted to NCBI under the accession number MT940221.

In vitro experiments examining Hu sheep GCs

GCs were cultured with DMEM/F12 in six-well plates at a density of 5×10^5 cells/well. After overnight culture (at 60–70% confluence), the medium was replaced with serum-free DMEM/F12 with the following treatments: (1) medium containing various concentrations of LH (0, 1, 2, 5, 10 IU/mL; Sansheng, Ningbo, China) for 4 h; (2) medium containing LH (5 IU/mL) at the indicated time points; (3) medium with or without LH (5 IU/mL) and U0126 (20 μM) for 4 h. (4) medium with or without prostaglandin (PGE₂, P0409; Sigma-Aldrich, Inc.; 500 ng/mL) and U0126 (S1102; Selleck, Houston, TX, USA; 20 μM) for 4 h. After incubation at 37°C in a humidified atmosphere with 5% CO₂, the cells were collected for further analyses.

CITED4 overexpression and knockdown were achieved using the pEX-4-CITED4 plasmid and siRNA, respectively, as well as the negative controls (pEX-4-NC and siRNA-NC),

which were synthesized by GenePharma (Shanghai, China). All siRNA sequences are listed in Table 3. GCs cultured to 60–70% confluence were transfected with pEX-4-CITED4 plasmid or siRNA, using Lipofectamine 3000, according to the manufacturer's instructions. After 48 h, the transfected cells were collected for subsequent analyses. The medium samples were also obtained to evaluate E₂ and P₄ levels using ELISA kits (JEB-14992-1 and JEB-15484-1, respectively; Jin Yibai Biological Technology, Nanjing, China).

When GCs attained 60–70% confluence, pEX-4-NC and pEX-4-CITED4 plasmids as well as siRNA-NC and siRNA-CITED4 were transfected into GCs for 24 h. Subsequently, the medium was replaced with serum-free DMEM/F12 in the absence or presence of LH (5 IU/mL) or PGE₂ (500 ng/mL) for 4 h. GCs were then collected for gene expression assays.

Cell proliferation analysis

Proliferation of the transfected GCs was measured with the CCK-8 assay (Cell Counting Kit-8; KGA317; KeyGen, Nanjing, China) and EdU incorporation assay (5-ethynyl-2'-deoxyuridine; KGA337-500; KeyGen, Nanjing, China), according to the manufacturer's instructions. Briefly, the transfected GCs were treated with the CCK-8 reagent (10 μL/well) and incubated for 2 h at 37°C. The optical density was measured at 450 nm using a spectrophotometer (Thermo Fisher Scientific Inc.). For EdU analysis, five areas were selected from each replicate. Fixed and stained cells were visualized with

Table 3 The siRNA sequences of CITED4 and the normal control.

Items	Primer sequence (5'–3')	
	Sense	Antisense
siRNA-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
siRNA-CITED4	AGAGCGAGUUCGACUGCUUTT	AAGCAGUCGAACUCGCUCUTT

Apoptosis and cell cycles were analyzed by flow cytometry (BD Biosciences) using an Annexin V-APC/7-ADD apoptosis detection kit (KGA1025; KeyGen, Nanjing, China) and propidium iodide (PI; 550825; BD Pharmingen, San Diego, CA, USA), respectively. All procedures were conducted according to the manufacturer's instructions.

Immunohistochemistry was performed on Hu sheep ovaries, based on our previous protocol (Yao *et al.* 2017a). Rabbit anti-CITED4, -CBP, -C/EBP α , and -C/EBP β were used as primary antibodies, and goat anti-rabbit IgG was used as the secondary antibody. The negative controls were incubated with Tris-buffered saline instead of primary antibodies. All sections were stained with diaminobenzidine (DAB; AR1026; Boster, Wuhan, China) and examined with a light microscope (Nikon).

Total RNA was extracted using TRIzol reagent (Invitrogen), following the manufacturer's instructions. Subsequently, cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (RR047A; Takara). Then, qRT-PCR was performed with SYBR Green Master Mix (Q711-02; Vazyme, Nanjing, China) using the ABI 7500 Real-Time PCR System (Applied BioSystems), following the manufacturer's protocol. Relative gene expression levels were quantified using the $2^{-\Delta\Delta CT}$ method. GAPDH was used as an internal control. Primers are listed in [Table 2](#).

To detect protein expression, western blot was performed based on our previous protocol with a minor modification (Yao *et al.* 2017a). Briefly, the total protein was extracted using cell protein extraction reagent containing phenylmethanesulfonyl fluoride and phosphatase inhibitors. Protein concentrations were determined with a BCA protein assay kit (P0010; Beyotime, Shanghai, China). For each replicate, 15 µg of total protein was separated on a 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. Subsequently, the membranes were blocked with 5% fat-free milk for 2 h at room temperature (RT) and incubated at 4°C overnight with primary antibodies (rabbit anti-CITED4, -C/EBPβ, -PCNA, -BAX, -GAPDH and -β-actin). After washing with Tris buffer solution containing 0.05% Tween 20, the membranes were incubated with the secondary antibody at RT for 1 h. The immunoreactions were visualized with an ECL detection system (Fujifilm, Tokyo, Japan). The chemiluminescence intensity of protein bands was quantified with the Image J software. Of note, the C/EBPβ antibody was a polyclonal antibody that recognized both long and short isoforms of C/EBPβ.

Each experiment included three independent samples, and was repeated three times. Statistical analyses were performed with SPSS (version 19.0 for Windows). Differences between groups were statistically analyzed using one-way ANOVA followed by Tukey's *post hoc* test. Data are expressed as mean $\pm$ S.E.M. *P*-values < 0.05 were considered significant.

Sequence analysis of the cDNA fragment (989 bp) demonstrated that CITED4 coding sequence comprised 558 bp and encoded a 185-amino acid protein (Fig. 1A).

As shown in Fig. 1B, alignment of the predicted protein with homologous proteins from other species demonstrated high homology with goat (98.40%), bovine (98.40%), and human (86.10%) sequences and moderate sequence identity with mouse sequences (72.19%).

CITED4/CBP pathway proteins are primarily present in ovarian GCs and in corpora lutea of Hu sheep

To understand the role of CITED4/CBP pathway proteins in the ovary of Hu sheep, immunohistochemistry was performed. As shown in Fig. 2A, CITED4 (a, b), CBP (d, e), C/EBP α (g, h), and C/EBP β (k, l) were predominantly present in GCs of antral follicles; meanwhile, C/EBP α (f) and C/EBP β (g) were observed following the primordial follicle stage. Positive signals for CITED4 (c), CBP (e), C/

EBP α (i), and C/EBP β (m) were also detected in the luteal cells. No positive signals were observed in the negative controls (n-q). CITED4 mRNA expression was higher in the ovaries and corpora lutea than in the hypothalamus, pituitary glands, uterine horn, and oviducts (Fig. 2B). The highest expression of CITED4 mRNA was detected in 7–8 mm corpora lutea, while the lowest expression was observed in 5–6 mm corpora lutea (Fig. 2C).

CITED4 is a downstream target of the ERK1/2 pathway in Hu sheep GCs

Next, we used Hu sheep GCs as a model to evaluate the effect of LH on CITED4 expression. Results showed that CITED4 mRNA expression increased with increasing LH concentrations in culture medium (Fig. 3A; $P < 0.05$).

Figure 2 CITED4/CBP pathway proteins are primarily present in Hu sheep GCs. (A) Localization of CITED4 (a–c), CBP (d and e), C/EBP α (f–i), and C/EBP β (g–m) in Hu sheep ovaries was detected by immunohistochemistry. Negative control (n–q). All sections were stained with DAB. Scale bars: 20 μ m or 50 μ m. GCs, granulosa cells; LCs, luteal cells. (B and C) CITED4 mRNA expression in Hu sheep female reproductive organs (B) and corpora lutea of various sizes (C) was analyzed with qRT-PCR. Data are presented as mean $\pm$ S.E.M., and different superscript letters (a–c) represent significant differences ($P < 0.05$). The ratio above each column represents the mean mRNA expression levels of CITED4 between experiment and control groups. Each experiment included three independent samples and was repeated three times.

Figure 3 CITED4 is a downstream target of the ERK1/2 pathway in Hu sheep GCs. (A) qRT-PCR and (B) Western blot results evaluating CITED4 expression in GCs after treatment with various LH concentrations for 4 h. (C) qRT-PCR results of CITED4 mRNA expression in GCs at different intervals after LH (5 IU/mL) treatment. (D) Western blot results for ERK1/2, p-ERK1/2, and C/EBPβ protein expression in GCs after LH treatment for 4 h. (E) qRT-PCR results for CITED4 mRNA expression in GCs with or without the administration of LH (5 IU/mL) and U0126 (20 μM) for 4 h. (F, G and H) qRT-PCR results for CITED4, AREG, and EREG mRNA expression in GCs with or without the administration of prostaglandin (PGE2, 500 ng/mL) and U0126 (20 μM) for 4 h. Data are presented as mean ± s.e.m., and different superscript letters (a–d) represent significant differences ($P < 0.05$). The ratio above each column represents the mean mRNA or protein expression levels of genes between experiment and control groups. Each experiment included three independent samples and was repeated three times.

This association was also confirmed at the protein level (Fig. 3B). CITED4 mRNA expression increased remarkably from 2–24 h after LH treatment, compared with that in the untreated controls (Fig. 3C; $P < 0.05$); the maximum increase in expression was reached 4 h after LH treatment. LH treatment also increased the protein expression of ERK1/2, p-ERK1/2, and C/EBPβ in GCs (Fig. 3D).

We further investigated whether direct inhibition of ERK1/2 using U0126 could block CITED4 and LH-target genes expression in GCs. Notably, U0126 significantly inhibited CITED4 mRNA expression, which was attenuated by LH (Fig. 3E; $P < 0.05$). Meanwhile, PGE2, the physiological intra-follicular mediator of LH effects, induced CITED4 mRNA expression in the GCs. However, its effects were blocked by U0126 (Fig. 3F; $P < 0.05$). U0126 also significantly weakened PGE2 influence on AREG and EREG mRNA expression (Fig. 3G and H; $P < 0.05$).

CITED4 is required for LH-target gene expression in GCs

The pEX-4-CITED4 plasmid was constructed by cloning CITED4 into the pEX-4 vector (Fig. 4A, B and C). CITED4 mRNA and protein levels were enhanced by CITED4 overexpression and were attenuated by CITED4 knockdown (Fig. 4D, E, F and G; $P < 0.05$).

Next, we investigated whether CITED4 is required for LH-triggered target gene expression in Hu sheep GCs. As shown in Fig. 5A, B, C, D, E, F, G, H, I, J, K, L, M and N, with the exception of the *HAS2* and *PTX3*

genes ($P > 0.05$), addition of LH to the culture media significantly enhanced the mRNA expression of *CITED4* and ovulation-related genes (*EREG*, *AREG*, *PTGS2*, and *TNFAIP6*) in GCs, which are considered LH-target genes ($P < 0.05$). Meanwhile, with the exception of *TNFAIP6* ($P > 0.05$), mRNA expression of LH-target genes was upregulated by CITED4 overexpression and downregulated by CITED4 knockdown ($P < 0.05$). Moreover, CITED4 overexpression moderately increased GC responses to LH by significantly increasing LH-target gene mRNA expression, whereas the opposite changes were observed with CITED4 knockdown ($P < 0.05$). The mRNA levels of CITED4 and LH-target genes in the GCs (*AREG*, *EREG*, *HAS2*, and *TNFAIP6*) increased significantly 4 h after PGE2 treatment (Fig. 5O, P, Q, R and S; $P < 0.05$). In contrast, knockdown of CITED4 in GCs significantly weakened PGE2-induced LH-target gene expression (Fig. 5O, P, Q, R and S; $P < 0.05$).

CITED4 effect on Hu sheep GC proliferation

EdU and CCK8 assays were conducted to characterize CITED4 effects on the *in vitro* GC proliferation. CITED4 overexpression and knockdown moderately promoted and inhibited GC proliferation, respectively (Fig. 6A, B and C; $P < 0.05$). The cell cycle progression of CITED4-overexpressing GCs was in line with its effects on proliferation, as demonstrated by the significant decrease in S phase and increase in G2/M phase percentages in transfected GCs (Fig. 6D; $P < 0.05$). However, no changes in GC cell cycle progression were observed with CITED4 knockdown (Fig. 6E; $P > 0.05$). CITED4 overexpression

Figure 4 Evaluation of CITED4 overexpression and knockdown efficiency in Hu sheep GCs. (A) The backbone of the CITED4 overexpression vector. (B) Restriction enzyme digestion of the pEX-4-CITED4 plasmid. (C) Representative micrographs of the overexpression vector transfected into Hu sheep GCs. (D, E, F and G) The efficiency of overexpression (D and E) and suppression (F and G) was determined with qRT-PCR and Western blot. Data are presented as mean $\pm$ S.E.M., and different superscript letters (a, b) represent significant differences ($P < 0.05$). The ratio above each column represents the mean mRNA or protein expression levels of genes between experiment and control groups. Each experiment included three independent samples and was repeated three times.

significantly upregulated CDK1 and Cyclin B1 mRNA expression in the GCs (Fig. 6F; $P < 0.05$), while CITED4 knockdown significantly downregulated the CDK1 mRNA expression (Fig. 6G; $P < 0.05$). However, transfection with CITED4 did not alter either the mRNA expression of CDK4 or CyclinD1 (Fig. 6F and G; $P > 0.05$). Additionally, proliferating cell nuclear antigen (PCNA) protein expression in the transfected GCs was significantly enhanced by overexpression and inhibited by knockdown of CITED4 (Fig. 6H; $P < 0.05$).

CITED4 effect on Hu sheep GC the apoptosis

To elucidate CITED4 role in GC apoptosis, flow cytometry, qRT-PCR, and western blot assays were performed. CITED4 overexpression and knockdown moderately inhibited and promoted apoptosis in GCs, respectively (Fig. 7A; $P < 0.05$). These results are in conjunction with the significant downregulation of BAX mRNA and protein expression following CITED4 overexpression and upregulation of that following CITED4 knockdown in GCs (Fig. 7B, C and D; $P < 0.05$). As shown in Fig.

7B, CITED4 overexpression significantly upregulated Bcl-2 mRNA expression, downregulated caspase8 mRNA expression, and decreased the BAX/Bcl-2 ratio ($P < 0.05$); no significant difference in caspase3 mRNA expression was observed ($P > 0.05$). However, although CITED4 knockdown had no effect on the gene expression of Bcl-2 (Fig. 7C; $P > 0.05$), it significantly increased the BAX/Bcl-2 ratio and upregulated the mRNA expression of caspase3 and caspase8 (Fig. 7C; $P < 0.05$).

CITED4 effect on steroidogenesis in Hu sheep GCs

We also assessed the effect of CITED4 on steroidogenesis. Results showed that P_4 concentrations were moderately increased by CITED4 overexpression and decreased by CITED4 knockdown in the GCs (Fig. 8A; $P < 0.05$); however, no effects were observed on the E_2 levels (Fig. 8B; $P > 0.05$). Knockdown of CITED4 significantly decreased the mRNA expression of StAR protein (StAR) and 3 beta-hydroxysteroid dehydrogenase/isomerase (3 β -HSD) in the GCs (Fig. 8C; $P < 0.05$). In contrast, overexpression of CITED4 significantly increased StAR

Figure 5 CITED4 is required for LH-target gene expression in GCs. (A, B, C, D, E, F, G, H, I, J, K, L, M and N) qRT-PCR results of mRNA expression of the indicated genes in GCs stimulated with or without LH (5 IU/mL) and CITED4 overexpression (A, B, C, D, E, F, G) or knockdown (H, I, J, K, L, M, N). (O, P, Q, R, S) qRT-PCR results for mRNA expression of the indicated genes in GCs stimulated with or without prostaglandin (PGE₂, 500 ng/mL) and CITED4 knockdown. Data are presented as mean $\pm$ S.E.M., and different superscript letters (a–d) represent significant differences ($P < 0.05$). The ratio above each column represents the mean mRNA expression levels of genes between experiment and control groups. Each experiment included three independent samples and was repeated three times.

mRNA expression (Fig. 8D; $P < 0.05$); however, no effect on the mRNA expression of 3 β -HSD was observed (Fig. 8D; $P > 0.05$).

Discussion

Culturing of GCs remains useful for understanding the molecular processes of follicular development, although suitable *in vitro* models for exploring ovarian functions are still limited (Skory *et al.* 2015). Here, we used GCs of antral follicles as a model to elucidate the

mechanism through which CITED4 influences Hu sheep GCs; however, the cultured GCs spontaneously convert into luteinized GCs during *in vitro* culture with serum (Tosca *et al.* 2010). Although this model may not be ideal for studying the mechanisms involved in follicular development, previous studies (Zhang *et al.* 2016, 2017, Yao *et al.* 2017b) have shown that this *in vitro* culture model can efficiently reflect GCs functions associated with proliferation, apoptosis, and steroidogenesis.

LH stimulation is critical for the regulation of folliculogenesis and ovulation (Filicori *et al.* 2002).

Figure 6 CITED4 regulates cell proliferation in Hu sheep GCs. (A) The percentage of cell viability determined with CCK8 assay. (B and C) Representative micrographs of EdU-incorporating cells. Nuclei were stained with Hoechst 33342. (D and E) Histograms represent the percentages of cell cycle distribution. (F and G) Relative expression of cell cycle-associated genes. (H) PCNA protein expression. Data obtained after CITED4 overexpression and knockdown in GCs are presented as mean $\pm$ S.E.M., and different superscript letters (a, b) represent significant differences ($P < 0.05$). The ratio above each column represents the mean mRNA or protein expression levels of genes between experiment and control groups. Each experiment included three independent samples and was repeated three times.

Moreover, during preovulatory follicular growth, FSH induces the expression of luteinizing hormone receptor (LHR) in GCs, and during advanced follicular development, the stimulation of mature GCs by LH mimics the action of FSH (Palermo 2007). It was suggested that CITED4 could be required for the expression of LH-induced target genes during ovulation in mice (Zhang *et al.* 2014). Nonetheless, limited information is available regarding CITED4 expression and its roles in sheep ovaries. CITED family proteins may interact with sequence-specific DNA-binding proteins and function as CBP/p300-dependent transcriptional co-activators (Yahata *et al.* 2002), mediating activities such as the binding of CITED4-CBP with C/EBP β to reinforce its transcriptional activity (Lee *et al.* 2010, Zhang *et al.* 2014). In this study, CBP, C/EBP α , and C/EBP β proteins were mainly present in ovarian GCs of all follicular stages, whereas CITED4 was detected in ovarian GCs of antral follicles. Thus, CBP, C/EBP α and

C/EBP β proteins may have critical roles throughout follicular development, although the potential role of CITED4 in GCs in follicular development may start with LHR expression and continue in the corpus luteum influenced by hCG stimulation through LHR (Casarini *et al.* 2017). This suggestion is consistent with previous studies that indicated high expression levels of CITED4, CBP, and C/EBP β in mouse ovaries and cultured GCs after hCG treatment (Zhang *et al.* 2014); similar results were observed for C/EBP α and C/EBP β in porcine GCs after LH or hCG treatment (Zhen *et al.* 2014).

Although the transfection of CITED4 in mice GCs indicated that CITED4 is indispensable for LH-induced ovulation-related processes (Zhang *et al.* 2014), no data are available to confirm whether that the expression of LH-triggered target genes is mediated by CITED4 in the GCs of ruminants. Herein, we demonstrated that treatment with LH or PGE2 significantly upregulated the mRNA or protein expression of CITED4 and other

Figure 7 CITED4 regulates cell apoptosis in Hu sheep GCs. (A) Percentage of apoptotic cells. (B and C) Relative expression of apoptosis-related genes. (D) BAX protein expression. Data obtained from CITED4 overexpression and knockdown in GCs are presented as mean $\pm$ S.E.M., and different superscript letters (a, b) represent significant differences ($P < 0.05$). The ratio above the each column represents the mean mRNA or protein expression levels of genes between experiment and control groups. Each experiment included three independent samples and was repeated three times.

ovulation-associated genes in GCs, whereas the inhibition of ERK1/2 (U0126) weakened these effects. These findings are consistent with previous studies (Ben-Ami *et al.* 2006, Yamashita *et al.* 2007, Fan *et al.* 2009, Zhang *et al.* 2014, Tang *et al.* 2017, Niringiyumukiza *et al.* 2018). Notably, CITED4 overexpression increased the responses of GCs to LH, whereas the effects of LH were blocked by silencing CITED4. These observations suggest that CITED4 is crucial for LH-induced ovulation-associated events in

sheep. In addition, the ERK1/2 cascade is involved in follicular development and ovulation, as it can mediate LH actions in rodent GCs during ovulation (Fan *et al.* 2009) and promote GC proliferation and survival in goats after LH treatment (Gupta *et al.* 2012). Consistent with this, ERK1/2 and p-ERK1/2 proteins levels in GCs were increased after LH treatment.

Follicular development is closely related to GC proliferation and apoptosis. The influence of CITED4

Figure 8 CITED4 regulates P₄ synthesis in Hu sheep GCs. (A) P₄ and (B) E₂ levels in the culture medium. (C and D) StAR and 3β-HSD mRNA expression. Data obtained from CITED4 overexpression and knockdown in GCs are presented as mean $\pm$ S.E.M., and different superscript letters (a, b) represent significant differences ($P < 0.05$). The ratio above each column represents the mean mRNA expression levels of genes between experiment and control groups. Each experiment included three independent samples and was repeated three times.

on follicular development could be associated with C/EBP β , which regulates GC apoptosis and proliferation (Zhen *et al.* 2014, Yuan *et al.* 2018), as bonding between CITED4/CBP and C/EBP β (as a triple complex) induces the expression of its target genes (Lee *et al.* 2010, Zhang *et al.* 2014). In the absence of LH stimulation, our results demonstrated that CITED4 expression is positively correlated with proliferation and negatively correlated with apoptosis of GCs, which could indicate LH-independent responses of CITED4. In addition, previous studies have reported that CITED4 knockdown inhibits the proliferation of colorectal cancer cells (Rogers *et al.* 2016) and embryonic stem cell derived cardiomyocytes (Miake *et al.* 2017). However, the inhibition of colorectal cancer cell proliferation following CITED4 knockdown was not found to be associated with induction of apoptosis (Rogers *et al.* 2016). This discrepancy between the previous results and our study can be attributed to the differences in cell types and transfection conditions. Nevertheless, although the influence of CITED4 on the proliferation and apoptosis of GCs was statistically significant, only slight changes occurred, which is due to the absence of LH stimulation. Thus, the mechanisms of CITED4 in regulating GC proliferation and apoptosis should be explored further.

Cell cycle regulation represents a major regulatory mechanism of cell growth, which is typically regulated by several types of cyclins, such as cyclin-dependent kinase (CDK) and cyclin-dependent kinase inhibitor (CKI) (Aylon & Kupiec 2005, Buck *et al.* 2009, Lavi *et al.* 2011). CITED1 downregulation suppressed cell proliferation by inducing cell cycle arrest in the G2/M phase, which was accompanied by downregulation of CDK1 and CyclinB1 expression (Jiang *et al.* 2016). Consistent with these observations, our results demonstrated that CITED4 overexpression induced cell cycle transition from S to G2/M phase, which was accompanied by the upregulation of CDK1 and Cyclin B1 expression. These results supported the hypothesis that activation of the CyclinB1-CDK1 complex is required for cells to pass the G2/M restriction point (Sun *et al.* 2019). CITED4 knockdown did not affect cell cycle distribution or the mRNA expression of Cyclin B1, CKD4, and Cyclin D1 in GCs, since the activation of Cyclin D1-CDK4 complexes is required for cells to pass the G1/S restriction point (Dong *et al.* 2001). However, CITED4 knockdown can inhibit cell proliferation by moderately inhibiting the cell cycle at the G2/M phase in colorectal cancer cells (Rogers *et al.* 2016). This difference between our results and those of the previous study can be attributed to variations in cell types and treatment methods.

Apoptosis is a precisely controlled process regulated by members of the caspase and Bcl-2 families. In the intrinsic apoptosis pathway, pro-apoptotic factors in mitochondria are released following cell cycle arrest or damage to cellular DNA, leading to apoptosis induction

by the activation of caspase3. Meanwhile, caspase8 activation by death receptors in the extrinsic apoptosis pathway can activate caspase3 directly or indirectly via activation of Bid in the intrinsic apoptosis pathway (Kiraz *et al.* 2016). Bcl-2 plays a crucial role in the inhibition of apoptosis (Spampanato *et al.* 2012), and the ratio of BAX to Bcl-2 is typically regarded as an important indicator of apoptosis (Korsmeyer *et al.* 1993). Here, although CITED4 overexpression significantly affected the gene expression levels of BAX, Bcl-2, and caspase8, only a minor reduction was observed in the apoptosis rate, with no changes observed in caspase3 gene expression. CITED4 knockdown significantly increased the apoptosis rate and the gene expression levels of BAX, caspase8, and caspase3. Moreover, our findings demonstrate that the ratio of BAX/Bcl-2 was negatively correlated with CITED4 expression in GCs. Thus, low CITED4 expression might trigger the apoptotic cascade in Hu sheep GCs; meanwhile, CITED4 might regulate GC apoptosis via a mechanism initiated by the regulation of apoptosis-related genes, primarily BAX and caspase8.

Ovarian steroids play essential roles in mammalian folliculogenesis. Particularly, the steroidogenesis process in GCs is vital during follicular development (Sanchez *et al.* 2016). C/EBP β knockdown in porcine GCs significantly inhibited P₄ production (Mizutani *et al.* 2014, Zhen *et al.* 2014), which is consistent with our results demonstrating that CITED4 expression in GCs of Hu sheep antral follicles significantly induced P₄ secretion. P₄ biosynthesis is a complex process modulated by multiple proteins and enzymes including StAR and 3 β -HSD (Yao *et al.* 2017b), which can positively upregulate the production of P₄ (Mizutani *et al.* 2015). In this study, CITED4 knockdown in GCs significantly downregulated the mRNA levels of StAR and 3 β -HSD. C/EBP β knockdown in porcine GCs significantly was shown to inhibit the production of P₄ and E₂ (Zhen *et al.* 2014). In the present study, we found that CITED4 expression demonstrated no significant effect on E₂ secretion. This finding is likely due to the absence of CYP17A1 (Cytochrome P450 17A1) in sheep GCs, and the fact that we did not add androgen precursor to the culture media (Padmanabhan *et al.* 2014). These observations demonstrated that CITED4 may be involved in the regulation of steroidogenesis in Hu sheep GCs.

In conclusion, this study demonstrated that CITED4 is primarily expressed in ovarian GCs of antral follicles, and was induced after LH treatment. CITED4 overexpression significantly enhanced the expression of ovulation-related gene in GCs following LH treatment, suggesting that CITED4 overexpression moderately augmented the responses of GCs to LH. In contrast, knockdown of CITED4 in GCs decreased PGE2-induced LH-target genes. Moreover, the stimulation of CITED4 mRNA expression by LH or PGE2 was blocked by ERK1/2 inhibition (U0126), suggesting that CITED4 is a downstream target of the ERK1/2 pathway in sheep GCs. It was also determined that

CITED4 could regulate GC proliferation, apoptosis, and P₄ production in Hu sheep by regulating the expression of genes associated with the cell cycle, apoptosis, and steroid hormones, thus revealing that CITED4 plays an important role in follicular development. Overall, the current study sheds light on the biologic significance of CITED4 in Hu sheep GCs; however, more studies are required to further explore these mechanisms.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

X Y: Conceptualization, Investigation, Visualization, Writing, Original Draft. M A S: Validation, Formal analysis, Data Curation. X S: Conceptualization, Investigation, Visualization. Z W: Validation, Formal analysis, Review and Editing. F M: Visualization; Resources. X L: Resources. Y B: Resources. Y Z: Review and Editing. Y F: Resources. F W: Supervision, Funding acquisition. All authors have read and approved the final manuscript.

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