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Knockdown of CEBPβ by RNAi in porcine granulosa cells resulted in S phase cell cycle arrest and decreased progesterone and estradiol synthesis

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ABSTRACT

Cultured ovarian granulosa cells (GCs) are essential models to study molecular mechanisms of gene regulation during folliculogenesis. CCAAT enhancer binding proteins β (CEBPβ) has been identified in the ovary and is critical for follicular growth, ovulation and luteinization in mice. In the present study, hormonal treatment indicated that luteinizing hormone (LH) and exogenous human chorionic gonadotropins (hCG) significantly increased the expression of CEBPβ in porcine GCs. By RNAi-Ready pSIREN-RetroQ-ZsGreen Vector mediated recombinant pshRNA vectors, CEBPβ gene was successfully knocked down in porcine GCs, confirmed by mRNA and protein level analyzed by real time PCR and western blot, respectively. We further found that knockdown of CEBPβ significantly increased the expression of p-ERK1/2. Furthermore, CEBPβ knockdown arrested the GCs at S phase of cell cycle, but had no effects on cell apoptosis. More importantly, it markedly down regulated the concentration of estradiol (E2) and progesterone (P4) in the culture medium. To uncover the regulatory mechanism of CEBPβ knockdown on cell cycle and steroids synthesis, we found that the mRNA expression of bcl-2 (anti-apoptosis), StAR and Runx2 (steroid hormone synthesis) was up-regulated, while genes related to apoptosis (Caspase-3 and p53), hormonal synthesis (CYP11A1) and cell cycle (cyclinA1, cyclinB1, cyclinD1) were down-regulated, suggesting that knockdown of CEBPβ may inhibit apoptosis, regulate cell cycle and hormone secretions at the transcriptional level in porcine GCs. Furthermore, knockdown of CEBPβ significantly increased the expression of PTGS2 and decreased the expression of IGFBP4, Has2 and PTGFR which are important for folliculogenesis in porcine GCs. In conclusion, this study reveals that CEBPβ is a key regulator of porcine GCs through modulation of cell cycle, apoptosis, steroid synthesis, and other regulators of folliculogenesis.

1. Introduction

Mammalian folliculogenesis is involved in follicular growth and follicular atresia. The follicular growth included the proliferation, cell cycle control and differentiation of GCs. However, only a few follicles undergo ovulation and the majority of follicles are lost before ovulation by atresia. This degenerative process is initiated or caused by granulosa cell apoptosis [1]. GCs produce steroids, sense follicle stimulating hormone (FSH) and LH in ovarian micro-environment and promote growth of delicate oocyte [1,2]. Therefore, cultured ovarian GCs are essential models to study molecular mechanisms of gene regulation during folliculogenesis.

Ovulation and luteinization are triggered by a preovulatory surge of LH and are obligatory for fertility in all mammals. The ovulatory LH triggers a series of dramatic morphological and physiological changes in the preovulatory follicles, facilitating the formation of corpus luteum [3,4], and activates multiple signaling pathways in GCs of preovulatory follicles, including the cAMP/protein kinase A (PKA) [5] and the epidermal growth factor (EGF) receptor (EGFR)/RAS/ERK1/2 (MAPK3/1) signaling cascade [6,7]. Substantial evidence has accumulated to indicate the essential role of the ERK1/2 cascade in ovulation, including cumulus cell–oocyte complex (COC) expansion [6,8], oocyte maturation [6,9], and follicle rupture [6,10]. Targeted disruption of ERK1/2 in mouse GCs resulted in oocyte maturation, ovulation and

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luteinization failure [6]. CEBP α and CEBPβ have been found to mediate specific cell fate decisions initiated by ERK1/2 activation and transcriptional regulation of genes that control luteinization [4,11].

CEBPs are family of six transcription factors, and at least two of them (CEBPα, CEBPβ) are reported in the ovary [12]. Both CEBPα and CEBPβ are expressed in follicular GCs, and are dynamically regulated by gonadotropins [4,6]. In particular, the abundance of CEBPβ protein is observed in preovulatory follicles (8–10 mm) in porcine and bovine GCs [13]. A previous study documented that GC-specific CEBPα$^{gc-/-}$ mice exhibited normal follicular development and fertility, indicating that disruption of CEBPα gene alone in the GCs is not sufficient to mimic the global effects of ERK1/2 depletion. The phenotype of the CEBPβ$^{gc-/-}$ mice was more severe [4] than that of depleting CEBPα alone, and CEBPα/β$^{gc-/-}$ mice are completely infertile [5]. These results show that these transcription factors have overlapping functions and CEBPβ appears to play a major role that controls LH-mediated ovulation and luteinization. In previous studies, CEBPβ has been shown to regulate proliferation of various cell types in different organs [14–17]. In particular, ovarian stage specific fluctuation in CEBPβ expression appears to correlate with follicular differentiation and increased steroidogenic capacity [4].

Although previous research have extensively studied the roles of CEBPβ in mouse ovarian using gene knockout approach, little information was obtained in porcine which is a very important economic animal to supply meat for human. Many research progresses obtained in mouse model could not be completely or directly applied in porcine for fertility improvement due to the species difference [4,6]. In order to improve the fertility and breedings of porcine, it is necessary to understand the regulatory mechanism of the reproductive process, especially the folliculogenesis of female pig. Although some information about CEBPβ have been inferred in porcine ovaries, such as the expression profile or the activity of CEBPβ during folliculogenesis and the transition period from follicle to corpus luteum [18], little is known about the roles of CEBPβ with regard to GCs apoptosis, cell cycle control, proliferation, and steroid hormone synthesis.

Because of the functional importance, GCs of follicles and their convenient isolation and constitution cultured is a well standardized model for an in-vitro study. In particular, gene expression analyses represent a useful approach to study genetic mechanisms underlying reproductive traits in vivo. Therefore, the aim of this study is to reveal the roles of CEBPβ in regulation of cell cycle, apoptosis, and steroid hormone biosynthesis in porcine GCs by RNAi-Ready pSIREN-RetroQ-ZsGreen Vector mediated recombinant pshRNA vectors. To further uncover the regulatory mechanism of CEBPβ on porcine GCs, real-time PCR was performed to analyze the expression of genes related to cell cycle, steroidogenesis or apoptosis. The response of CEBPβ to LH/hCG treatment and effects of CEBPβ knockdown on p-ERK1/2 were also studied in porcine GCs. Furthermore, genes associated with folliculogenesis and luteinization in mice were also analyzed after CEBPβ knockdown to reveal the effects of CEBPβ during folliculogenesis and luteinization. The results of this study would help to understand the regulatory functions of CEBPβ in porcine GCs and provide important information for the regulatory mechanism of folliculogenesis and ovulation in pigs.

2. Materials and methods

2.1. Chemicals and solution

Polyclonal rabbit anti-CEBPβ (sc-746), polyclonal rabbit anti-ERK2 (sc-154), and monoclonal mouse anti-p-ERK1/2 (sc-7383) antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Lipofectamine® LTX & Plus reagent and TCM199 were purchased from Invitrogen (Carlsbad, USA). Apoptosis detection kit (Annexin V-APC/7-AAD) and cell cycle detection kit were purchased from KeyGEN (China), whereas, large scale plasmid extraction kit and RNAprep pure Cell Kit were acquired from Tiangen biochemistry technology limited China. Fetal Bovine Serum (FBS) was purchased from Hyclone (USA). All other chemicals were purchased from Sigma-Aldrich unless stated otherwise.

Table 1
Target sequences of porcine CEBPβ.

Group	Target sequence (5′ - 3′)	Position cds
pshRNA-1	GTGGCCAACTTCTACTACG	73
pshRNA-2	CCAAGAAGACCGTGGATAA	791
pshRNA-3	GCACAGCGACGAGTACAAG	810
pshRNA-4	GATGCGCAACCTGGAGACG	882
pshRNA-negative	TGGACATAGGCGACGTGT	

2.2. Porcine GCs isolation, culture and treatment

Porcine ovaries were obtained from COFCO Company (Wuhan, China). Porcine GCs were isolated from antral follicles (5–8 mm) by needle puncture method and cultured in TCM199 medium supplemented with 10% FBS and 1% penicillin and streptomycin. The viability of GCs was determined by trypan blue dye. Cells were counted at each passage and plated at a concentration of 2×10^6 viable cells in 6-well plates for transfection. For protein extraction, cells were cultured in 35-mm culture dishes and later transfected with respective vectors. All cultures were carried out at 37 °C in a humidified atmosphere of 5% CO_2.

For hormonal treatment, porcine GCs were cultured in 6-well plate and cultured in medium with or without 10 mIU/ml of LH or 5 ng/ml of hCG. After 8 h or 14 h, cells were collected for protein and RNA extraction in order to detect the expression of CEBPβ by western blotting and real-time PCR, respectively.

2.3. Construction and transfection of recombinant pSIREN-RetroQ-ZsGreen Vectors

Briefly, four siRNA target sites were selected from coding sequence of porcine CEBP beta (CEBPβ, NM_001199889.1) according to the siRNA program at position 73, 791, 810 and 882 (Table 1). To obtain short hairpin RNA, a typical oligonucleotide that has 5 bases containing a restriction site at its 5′ end, 19 bases of sense strand, 7 to 9 bases of hairpin loop, 19 bases of antisense strand, 6 bases of terminator, and 6 bases corresponding to a unique HindIII restriction site (resulting in a total length of 65 bases), and 2 complementary oligonucleotides were synthesized. These were annealed and inserted into the BamHI and EcoRI sites of the RNAi-Ready pSIREN-RetroQ-ZsGreen Vector (BD Biosciences, Clontech, Mountain View, CA). The recombinant plasmids were designated as pshRNA-1, pshRNA-2, pshRNA-3, and pshRNA-4, respectively. A plasmid (pshRNA-negative) encoding a hairpin siRNA comprising a nonsense sequence that has not been found in the pig or human genomes was used as the negative control.

Plasmids of pshRNA-1, pshRNA-2, pshRNA-3, pshRNA-4 and pshRNA-negative were recycled using EndoFree plasmid kit (Tiangen, Beijing, China) and confirmed by sequencing. These vectors independently expressed a Zoanthus sp. Green fluorescent protein, which had been engineered for brighter fluorescence (excitation maximum = 496 nm; emission maximum = 506 nm), and as result, the transfected cells emitted green fluorescent (GFP).

Table 2
Sequences of primer pairs for quantitative real-time PCR.

Gene name	Primer sequences (5′ - 3′)		Length (bp)
CEBPβ	Forward	GGAGCCTGTCCACATCCTCG	174
	Reverse	CGCTCACGCCGGATCTTGTA	
Actin	Forward	TCTGGCACCACACCTTCT	130
	Reverse	TGATCTGGGTCATCTTCTCAC	
Bcl2	Forward	GGTCATGTGTGTGGAGAGCGTCAAC	190
	Reverse	CTTCAGAGACAGCCAGGAGAAATCA	
Casp3	Forward	TGGACTGTGGGATTGAGA	214
	Reverse	ACCCGAGTAAGAATGTGC	
P53	Forward	TCTGACTGTACCACCATCCACTA	128
	Reverse	ACAAACACGCACCTCAAAGC	
cyclinA1	Forward	GACGGCACCAACCACTA	256
	Reverse	ACTCAGGCAAGGCACAA	
cyclinB1	Forward	AATCCCTTCTTGTGGTTA	104
	Reverse	CTTAGATGTGGCATACTTG	
cyclinD1	Forward	TTACCTGGACCGCTTCTTG	155
	Reverse	GAGGCTTGATGGAGTTGTCG	
CYP11A1	Forward	GTCCCATTTACAGGGAGAAGCTCG	182
	Reverse	GGCTCCTGACTTCTTCAGCAGG	
StAR	Forward	CCCACCGCATGGTCGAGTAGTG	103
	Reverse	CGCCTCTGCAGCCAACTCATG	
Has2	Forward	ATTGCCACAGTAATCCAGC	158
	Reverse	TACAACACCGAGTAGAGGG	
PTGS2	Forward	AAGACAGATCAGAAGCGAGGAC	262
	Reverse	GACCAGGCACCAGACCAA	
Runx2	Forward	ATGCTTCATTCGCCTCACA	160
	Reverse	AAATTCTGCTTGCAGCCTTAA	
IGFBP4	Forward	ACCGCAACGGCAACTTCCA	89
	Reverse	TCCCGTCTTCCGGTCCACA	
PTGFR	Forward	AAGGCAGGTCTCATCATTT	141
	Reverse	TTGTTTCACAGGCATCCAG	

One day before transfection, $0.5–2 \times 10^5$ GCs were seeded in 6-well plate with 70–80% confluence. Six groups of GCs were prepared in total to transfect pshRNA-1, pshRNA-2, pshRNA-3, pshRNA-4, pshRNA-mix (1–4) or pshRNA-negative, respectively. The transfection procedure was performed using Lipofectamine™ LTX with Plus™ reagent (Invitrogen) according to manufacturer's instructions. After 6 h, transfection medium was changed into fresh growth medium without antibiotics. Cells were collected for RNA or protein extraction, and in some experiments, the culture medium was collected and preserved for detection of hormones after transfection for 48 h.

2.4. RNA extraction and real-time PCR

At 48 h after transfection, GCs were washed in PBS and total RNA was extracted using RNAprep pure cell kit (Tiangen, Beijing China). For the removal of residual genomic DNA, the samples were treated with DNaseI. The first-strand cDNA was synthesized using first strand cDNA synthesis kit (Thermo, Co. USA), and quantitative real-time PCR was carried out using SYBRGreen (QuantiFast SYBR Green PCR kit, QIAGEN). Specific PCR settings were used in a Bio-Rad LC480 real-time PCR system. To verify PCR product purity, samples were subjected through melting curve analyses after real-time PCR reactions. The threshold cycle (CT) numbers were calculated for the amplified cDNA for each investigated mRNA and for the housekeeping gene (β-actin) in each sample. The relative mRNA expression levels were estimated using the formula: $2 - \Delta\Delta CT$ [42].

To confirm the efficiency of CEBPβ knockdown, real-time PCR analysis was conducted to detect CEBPβ mRNA level after transfection. Relative mRNA expression of p53, bcl-2, Caspase-3 (apoptosis related genes), cyclin A1, cyclin B1, cyclin D1 (cell cycle related genes), CYP11A, StAR, Runx2 (Steroid hormone related genes) and Has2, IGFBP4, PTGFR, PTGS2, (CEBPβ regulated genes) were quantified by real-time PCR in GCs after transfection with pshRNA-2 and pshRNA-negative, respectively. All primer pairs used for real time PCR are summarized in Table 2.

2.5. Western blot analysis

Porcine GCs transfected with the recombinant RNAi vectors were scraped off from petri dish 48 h after transfection, washed in cold PBS and lysed in RIPA buffer (Santa Cruz, USA) containing protease inhibitor cocktail (Santa Cruz, USA). After 15 min incubation on ice, cells were centrifuged at 12,000 *g* for 5 min for removal of cellular debris. Total protein concentration was ascertained by BCA-assay (Pierce, Rockford, USA), and 20 μg of total protein was submitted to gel electrophoresis. Proteins were separated on a 12% polyacrylamide gel before transferring them to PVDF membranes (Millipore, Bedford, MA). After blocking in TBST [10 mM Tris (pH 7.5), 150 mM NaCl and 0.05% Tween 20] supplemented with 5% skim milk (Sigma-Aldrich) and 0.1% Tween 20 (Sigma-Aldrich), membranes were incubated overnight at 4 °C with anti-CEBPβ rabbit polyclonal antibody (1:200), anti-ERK2 rabbit polyclonal antibody (1:500), and anti-p-ERK1/2 mouse monoclonal antibody (1:200). After incubation with the primary antibodies, membranes were washed three times with TBST and then incubated for 1 h with 3000-fold diluted HRP labeled goat anti-rabbit or goat anti-mouse secondary antibodies (Boster Co, Wuhan, China) at room temperature. After incubation, the membrane was washed three times with pre-warmed TBST after 5 min interval. After washing, each membrane was developed using ECL Western Blotting detection system (Amersham Biosciences, Piscataway, NJ), and then exposed to X-ray film for visualization of the protein bands. After that, PVDF membrane was then stripped of bound antibodies and incubated with mouse monoclonal anti-β-actin antibody (1:1000 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for normalization. The band intensities were measured by using AlphaEaseFC software (AlphaInnotech, USA). For the negative control, the primary antibody was replaced by normal rabbit IgG or mouse IgG.

2.6. ELISA for measurements of steroid hormones

After transfection with pshRNAi-2 and pshRNA-negative, respectively, the culture medium was collected at 48 h to measure concentrations of E2 and Progesterone using the porcine ELISA kits. The sensitivity of E2 and Progesterone ELISA kit (Wuhan ColorfulGene biological technology Co., Ltd, China) was 1.8–50 pmol/L and 100–2000 pmol/L, respectively. The ELISA procedure was performed according to manufacturer's instructions.

2.7. Cell cycle analysis

Porcine GCs transfected with different RNAi vectors were harvested at 48 h after transfection, washed with PBS, fixed in ice-cold 70% ethanol overnight at 4 °C, washed again in PBS and stained using propidium iodide/RNase A solution at 37 °C in the dark chamber for 30 min. Flow cytometric analysis was conducted using a BD FACSCalibur (Becton, Dickinson and Company, USA) and ModFit LT for Mac V3.0 software. For each determination, a minimum of 20,000 cells was analyzed. All experiments were repeated five times independently.

2.8. Apoptosis analysis

After transfection, GCs were washed with PBS and then, harvested by digestion with trypsin without EDTA at 37 °C for 5 min. Cell apoptosis was probed with Annexin V-APC/7-AAD and later detected by flow cytometry (BDFACSCalibur, USA) according to the manufacturer's instruction (Apoptosis Detection Kit, KeyGEN, Nanjing, China). In this kit, viable cells show negative for Annexin V-APC and 7-AAD staining; early apoptotic cells stain positively with Annexin V-APC stain and remain negative for 7-AAD stain, while late apoptotic cells are double stained by Annexin V-APC and 7-AAD. Experiments were repeated five times independently.

2.9. Statistical analysis

Changes in the concentrations of E2 and progesterone were subjected to one-way ANOVA and level of significance was later determined by Duncan's multiple range tests. All data were analyzed using the General Linear Models Procedure of Statistical Analysis Systems (SPSS Inc., Cary, NC, USA). A value of $P < 0.05$ was considered to be significant. All data is represented as mean ± SEM of repeated experiments ($n = 3$).

3. Results

3.1. LH/hCG significantly increased the expression of CEBPβ in porcine GCs

LH surge triggers ovulation and luteinization, while CEBPα/β is a target protein which is regulated by ERK as the downstream of LH in the mouse model. However, whether this pathway is similar in porcine is still unclear. Therefore, the protein expression level of CEBPα/β was detected after LH or hCG treatment by western blot using CEBP antibody (sc-746, Santa Cruz) which could react with both of CEBPα (354 amino acids in pig) and CEBPβ (346 amino acids in pig), and the CEBPβ mRNA level by real-time PCR using CEBPβ specific primers. The results showed that CEBPα/β protein and CEBPβ mRNA were expressed in porcine GCs and the expression level was significantly increased after LH or hCG treatment (Fig. 1). These results confirm that CEBPβ is regulated by LH/hCG in porcine GCs, and provide evidence that CEBPβ may be a key regulatory factor in porcine ovary.

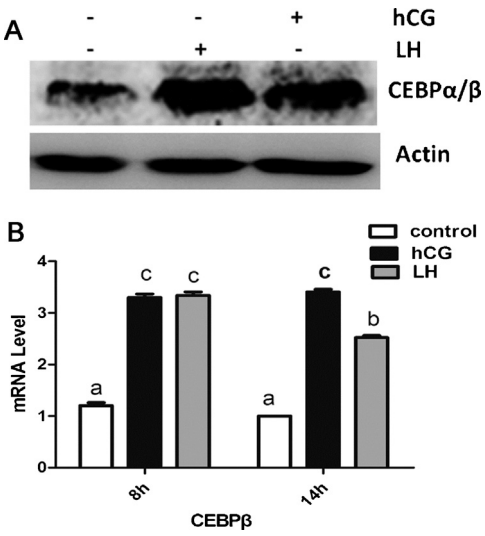

Fig. 1. *Upregulation of CEBPβ expression in porcine GCs after LH/hCG treatment.* (A) The expression of CEBPα/β protein in porcine GCs was detected by western blot after treatment with LH (10 mIU/ml) or hCG (5 ng/ml) for 8 h. (B) Transcription levels of CEBPβ gene in porcine GCs after treatment with LH or hCG for 8 h and 14 h, respectively. The expression of CEBPβ was significantly increased after LH and hCG treatment. "a" and "b" or "b" and "c" represents $P < 0.05$, "a" and "c" represents $P < 0.01$.

3.2. Identification and validation of CEBPB RNAi recombinant plasmids

CEBPβ RNAi recombinant plasmids were identified by restriction analysis and sequencing. There was a HindIII site at position 2456 in the pSIREN-RetroQ-ZsGreen plasmid, and another HindIII site was inserted in the hairpin fragment of the shRNA. Analysis of 2 fragments (2460 and 4173 base pairs, respectively) released from the recombinant plasmids through digestion with homologous restriction enzymes revealed that the all siRNAs were inserted correctly. These clones were further confirmed by sequencing (TaKaRa, Co., Japan).

3.3. CEBPβ was efficiently knocked down by the pshRNA-2

After construction and identification of the four pshRNA vectors, we intended to select the most efficient knockdown plasmid in porcine GCs. At 48 h after transfection, we first detected the transfection efficiency as measured by GFP in cultured GCs (Fig. 2A) and then, the mRNA level of CEBPβ was detected by real-time PCR (Fig. 2B), and the protein level was detected by western blot (Fig. 3A and B). The results showed that all four plasmids were able to knockdown the CEBPβ expression with pshRNA-2 having the greatest effect (~65%) as confirmed at transcriptional and translational levels.

3.4. Knockdown of CEBPβ altered the expression level of ERK2 and p-ERK1/2 in GCs

It has been reported that CEBPβ is one of the representative genes which is commonly regulated by ERK1/2 in the mouse ovary. To further test whether there is feedback regulation between ERK1/2 and CEBPβ in porcine model, protein expression of ERK2/p-ERK1/2 was analyzed by western blot. The results showed that expression of p-ERK1/2 was increased, whereas ERK2 was decreased after CEBPβ RNAi (Fig. 3A and C). These results suggest that both of these protein kinases are associated with the regulation of CEBPβ in porcine GCs.

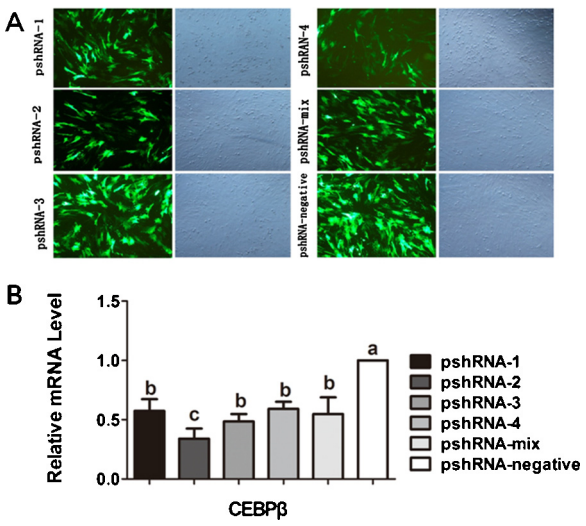

Fig. 2. *Transfection and knockdown efficiency of CEBPβ RNAi vectors.* (A) Six groups of CEBPβ RNAi recombinant plasmids were transfected in porcine GCs named as pshRNA-1, pshRNA-2, pshRNA-3, pshRNA-4, pshRNA-mix (1–4) and pshRNA-negative. After 48 h, the expression of GFP in the recombinant plasmids were shown, which implied that CEBPβ recombinant plasmids could be high efficiently expressed in porcine GCs. (B) Transcription level of CEBPβ gene in porcine GCs was detected after transfection with CEBPβ knockdown vectors, respectively, the ratio is relative to the pshRNA negative control group. All silencing vectors could efficiently knockdown the expression of CEBPβ, but the pshRNA-2 performed best, which was chosen for further study. "a" and "b" or "b" and "c" represents $P < 0.05$, "a" and "c" represents $P < 0.01$.

3.5. Knockdown of CEBPβ reduced the concentration of E2 and progesterone in porcine GCs culture medium

To assess the effect of CEBPβ silencing on steroid hormones, we measured the concentration of E2 and progesterone in culture medium 48 h post-transfection. The results showed that after transfection, the release of E2 and progesterone in GCs was significant lower in pshRNA-2 group ($P < 0.01$ and $P < 0.05$, respectively) than that of pshRNA-negative group (Fig. 4A).

To further confirm the lower release of these steroid hormones by CEBPβ knockdown, we analyzed mRNA expression of steroidogenic enzymes (CYP11A1, StAR, and Runx2) by real-time PCR. The results showed that down regulation of CEBPβ significantly decreased mRNA expression of CYP11A1, which is important for progesterone synthesis as a rate-limiting enzyme, while it significantly increased the mRNA expression of StAR (regulates cholesterol transport) and Runx2 (Fig. 4B). These results confirm that CEBPβ is involved in the regulation of steroidogenesis in porcine GCs.

3.6. Porcine GCs were arrested at S phase after knockdown of CEBPβ

To determine whether CEBPβ is involved in the regulation of cell cycle progression, we saturated transfected GCs with propidium iodide (PI) that usually stains the nuclear contents of a cell. Then, the cells were subjected to fluorescent activated cell sorter (FACS). The results showed that a significant number of GCs were arrested at S phase after CEBPβ knockdown ($P < 0.01$) compared with control group (Table 3).

In addition, to further confirm the results of cell cycle analysis, the transcriptional level of cell cycle factors (cyclin A1, cyclin B1,

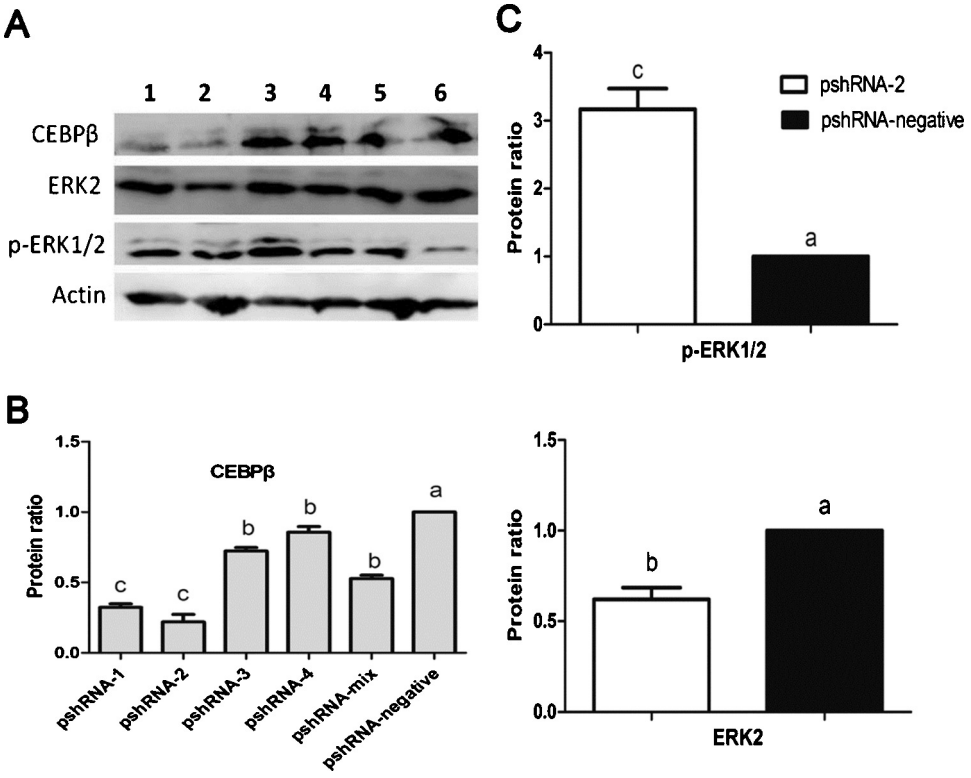

Fig. 3. *Effects of CEBPβ knockdown on the expression of ERK2 and p-ERK1/2 in GCs.* (A) Protein expression level of CEBPβ, ERK2 and p-ERK1/2 was detected by western blot in porcine GCs after transfection for 48 h. Lanes 1–6: proteins of porcine GCs after transfection for 48 h with CEBPβ interfering vectors: pshRNA-1, pshRNA-2, pshRNA-3, pshRNA-4, pshRNA-mix (1–4) and pshRNA-negative as the control, respectively. (B) The protein ratios of CEBPβ were analyzed according to the western blot in order to obtain the best CEBPβ knockdown vector for the further research and pshRNA-2 vector was selected. (C) The protein ratio of ERK2 and p-ERK1/2 in the pshRNA-2 transfection group relative to the pshRNA-negative control group was analyzed according to the western blot results. Actin was set up as a positive control. "a" and "b" or "b" and "c" represents $P < 0.05$, "a" and "c" represents $P < 0.01$.

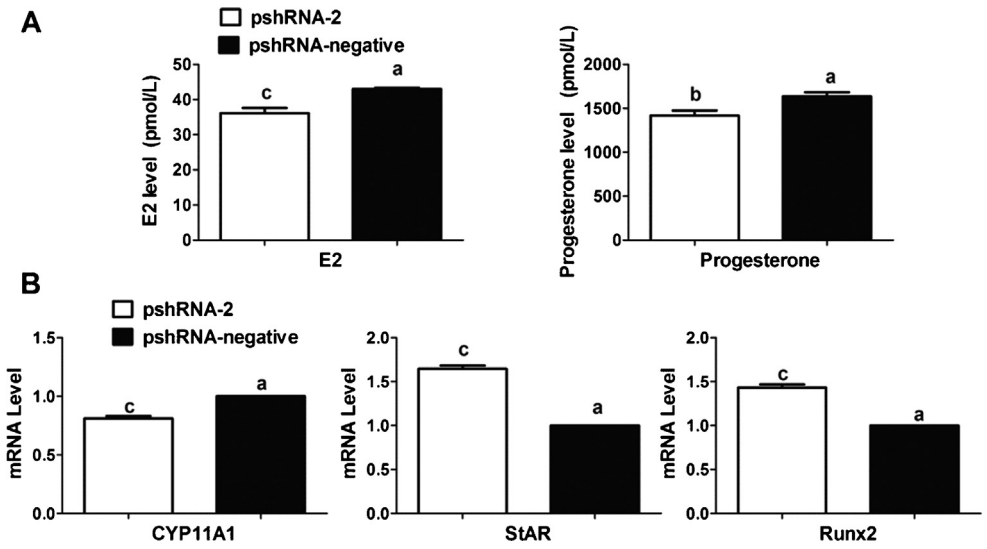

Fig. 4. *CEBPβ knockdown decreased the concentration of E2 and progesterone in GCs culture medium.* (A) Concentration of E2 and progesterone was significantly decreased in culture medium after transfection with pshRNA-2 group compared with pshRNA-negative group for 48 h. (B) Relative mRNA level of genes (CYP11A1, StAR and Runx2) related with hormonal secretion was compared in pshRNA-2 group and pshRNA negative control group. The results showed that the mRNA level of CYP11A1 was significantly decreased, while mRNA level of StAR and Runx2 was significantly increased in pshRNA-2 group than those in pshRNA-negative group "a" and "b" or "b" and "c" represents $P < 0.05$, "a" and "c" represents $P < 0.01$.

cyclin D1) was determined by real-time PCR. The results showed that after CEBPβ RNAi, a significant decrease in mRNA level of cyclinA1, cyclinB1, cyclinD1 was observed ($P < 0.05$ or $P < 0.01$) (Fig. 5A). These results show that CEBPβ appears to have a crucial role in the growth progression of GCs.

3.7. The effects of CEBPβ knockdown on porcine GC apoptosis

To elucidate the roles of CEBPβ in regulation of GCs apoptosis, we detected the exposure of phosphatidylserine on the cell surface with Annexin V-APC/7-AAD double staining in transfected GCs. The results showed that down-regulation of CEBPβ did not alter apoptosis compared to that of control (Table 4).

To further reveal the effects of CEBPβ knockdown on apoptosis, we quantified the mRNA expression of apoptotic inducers (Casp3 and p53), and anti-apoptotic factor (bcl-2) in these cells. CEBPβ RNAi significantly reduced the expression of casp3 and p53 mRNA level, but increased the mRNA level of bcl-2 (Fig. 5B). These results further confirmed that CEBPβ RNAi could not induce apoptosis in porcine GCs, but in turn, CEBPβ may act as an inducer of apoptosis or differentiation which is important for ovulation and luteinization in the ovary.

Table 3
Analysis of cell cycle by flow cytometry in porcine GCs 48 h post transfected with pshRNA-2 and pshRNA-negative (mean ± SEM, $n = 3$).

	G1 (%)	S (%)	G2 (%)
pshRNA-2	78.82 ± 0.89b	13.46 ± 3.22c	7.71 ± 3.67
pshRNA-negative	81.88 ± 0.38a	1.08 ± 0.71a	17.04 ± 1.05

All results were evaluated by one-way ANOVA. "a" and "b" indicates level of significance in columns ($P < 0.05$), "a" and "c" indicates level of significance in columns ($P < 0.01$).

Table 4
Measurement of porcine GCs apoptosis there were significantly less apoptotic cells and more vital cells in the pshRNA-2 group compared to those of pshRNA-negative (mean ± SEM, $n = 3$).

	Live cells (%)	Apoptotic cells (%)
pshRNA-2	93.83 ± 0.20	6.28 ± 0.197
pshRNA-negative	92.95 ± 0.85	6.30 ± 0.77

3.8. Silencing of CEBPβ altered the expression profile of genes involved in folliculogenesis in porcine GCs

We further studied the expression profile of genes (Has2, IGFBP4, PTGFR and PTGS2) which have been identified to relate with folliculogenesis, ovulation, and luteinization in mice, to reveal their potential involvement of CEBPβ in porcine ovary by real-time PCR. The mRNA levels of Has2 (Hyaluronan synthase 2), IGFBP4 (insulin-like growth factor binding protein 4), PTGFR (PGF2α receptor), PTGS2 (prostaglandin-endoperoxide synthase 2) were changed after CEBPβ knockdown. The results showed that silencing of CEBPβ significantly decreased the mRNA level of Has2, IGFBP4 and PTGFR ($P < 0.01$), but increased the mRNA level of PTGS2 ($P < 0.05$) (Fig. 6), suggesting an important role of CEBPβ on gene expression during folliculogenesis, ovulation, and luteinization in porcine ovary.

4. Discussion

Previous studies have demonstrated that CEBPβ, a potential mediator of ERK1/2, is the key regulatory factor in governing ovulation and luteinization in mice. Many common genes were down-regulated, such as CYP11A1, StAR, Runx2 and others (Has2 (cumulus expansion related), PTGFR (inducer of luteolysis) and IGFBP4 (follicular growth related)) in CEBPα/β$^{gc-/-}$ cells and ERK1/2$^{gc-/-}$ cells in mice [4,6]. But little information was obtained in porcine about the roles of CEBPβ with regard to GCs apoptosis, cell cycle control, proliferation, and steroid hormone synthesis. Therefore, we detected the function of CEBPβ in the porcine GCs in this study. The results showed that CEBPβ was involved cell cycle control, steroids synthesis and gene expression regulation in porcine GCs, indicated that CEBPβ is an important regulator in porcine ovary.

An important aspect of our study is to detect the response of CEBPβ to ovulatory hormone (LH) in porcine GCs. The amount of CEBPα/β protein and CEBPβ mRNA was significantly increased in porcine GCs after LH/hCG treatment, suggesting that CEBPβ can be up-regulated by LH/hCG. To further test whether there is a feedback regulation between ERK1/2 (upstream regulator of CEBPβ in mice) and CEBPβ, the expression of ERK2 and p-ERK1/2 was detected by western blot after CEBPβ knockdown. The results

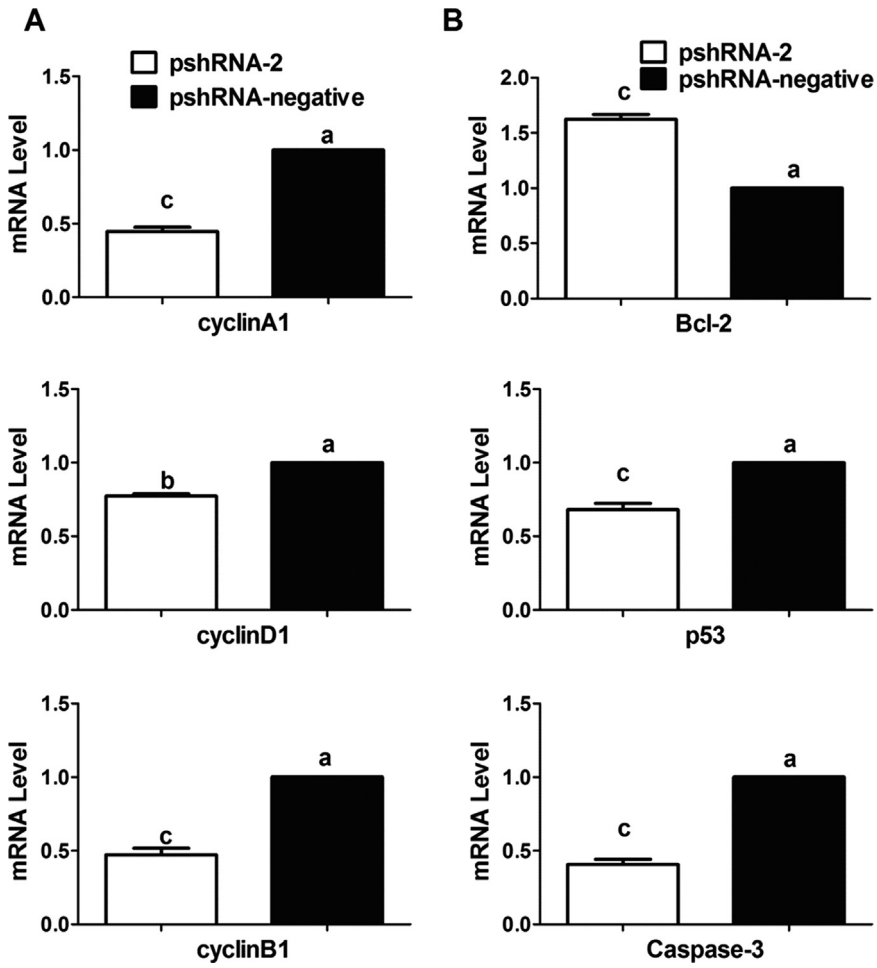

Fig. 5. *Effects of CEBPβ knockdown on the mRNA expression of genes related to cell cycle and apoptosis.* (A) The mRNA level of cell cycle related genes, cyclin A1, cyclin D1, and cyclin B1, all were decreased in pshRNA-2 transfection group compared with pshRNA negative control group. (B) Whereas anti-apoptotic factor bcl-2 was increased, apoptotic inducers p53 and Caspase-3 were decreased in pshRNA-2 transfection group compared with pshRNA negative control group. These results implied that silencing of CEBPβ affected porcine granulosa cell apoptosis and cell cycle. $P < 0.05$ as significant.

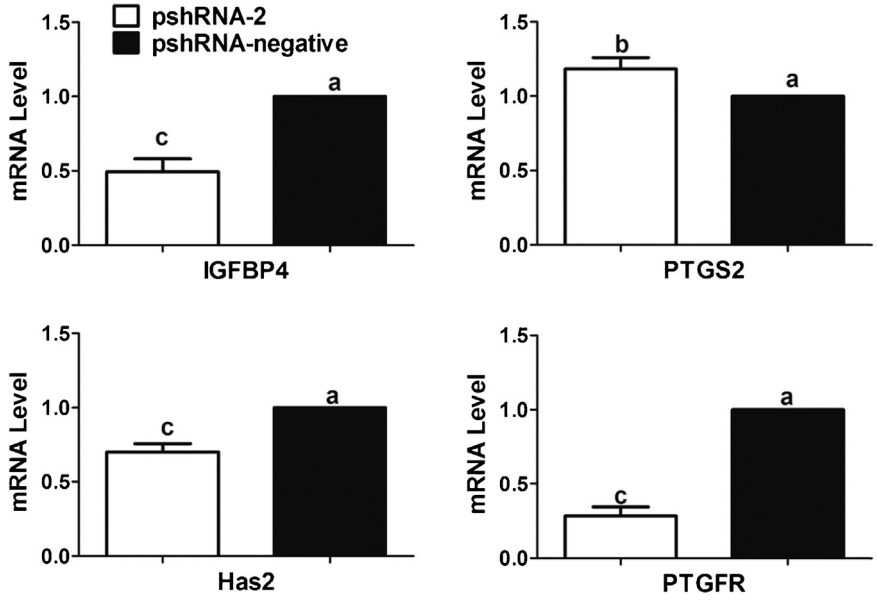

Fig. 6. *Effects of CEBPβ knockdown on the mRNA expression of genes related to folliculogenesis and luteinization.* The results showed that the mRNA expression level of Has2, IGFBP4 and PTGFR were all decreased, and the mRNA level of PTGS2 was increased in pshRNA-2 transfection group compared with pshRNA negative control group for 48 h. These results implied CEBPβ might be involved in the regulation of folliculogenesis, ovulation, and luteinization through regulation of these genes expression in porcine GCs. $P < 0.05$ as significant.

showed that the transient down regulation of CEBPβ expression resulted in increased expression of p-ERK1/2 along with decreased expression of ERK2, which implies that down-regulation of CEBPβ can affect the expression of ERK2 and the phosphorylation level of ERK1/2 in porcine GCs. These results indicate that CEBPβ may serve as a key regulatory factor in the regulation of LH and ERK1/2-mediated ovulation and luteinization in porcine, similar with that revealed in mice [4].

Follicular development is a complex biological process which is regulated by various factors from GCs, especially progesterone (P4), and 17b-estradiol (E2) [18–20]. Previous studies have shown that testosterone supplement could facilitate the synthesis of estradiol in cultured granulosa cells [21,22]. However, the primary cultured granulosa cells were also able to synthesize and secrete estradiol in other reports by supplement with 10% FBS [23–25]. We predicted that the progesterone could be directly converted into estradiol by P450arom and then 17β-HSD-1, without via testosterone to estradiol by 17β-HSD-1 and then P450arom.

In our culture system, CEBPβ silencing significantly reduced the concentration level of progesterone and estradiol in the primary cultured GC culture medium supplement with 10 % FBS, compared with the control group. The lower level of these two hormones might be due to the lower mRNA level of CYP11A1 caused by CEBPβ knockdown, which is responsible for initiation of steroidogenesis cascade in the ovary [26]. However, mRNA level of StAR was up-regulated after knockdown of CEBPβ in porcine GCs. We presume that in response to lower hormonal synthesis and lower level of CYP11A1, StAR may be promoted to translocate cholesterol from relatively sterol rich outer mitochondrial membrane to the relatively cholesterol-poor inner mitochondrial membrane, with an increasing expression pattern response to CEBPβ knockdown [26–28]. Recently, Runx2 transcription factor was found highly expressed in cumulus–oocyte complexes (COCs) and GCs of periovulatory ovaries in mice [29]. This finding suggests that Runx2 plays a crucial role in regulating periovulatory gene expression during ovulation and/or luteal development. In human ovaries, Runx2 expression is negatively associated with E2 level [30], which is consistent with the present study as an increase in Runx2 expression and a decreased estradiol were detected after knockdown of CEPBβ in porcine GCs.

The progresses of cumulus expansion, follicular growth and luteinization were regulated by many genes, we also selected some of those down-regulated genes (Has2, PTGFR and IGFBP4) in mice CEBPα/β$^{gc-/-}$ cells and PTGS2 (which was not affected by CEBPα/β knockout but important for luteinization) to test the regulatory function of CEBPβ in porcine GCs. Has2, PTGFR and PTGS2 are LH responsive genes and highly expressed in preovulatory follicles and cumulus cells [29,31]. IGFBP4, a potent inhibitor of WNT signaling [32], regulates luteinization and follicular growth through IGF signaling [33]. Although the localization of PTGS2 protein in granulosa cells and cumulus–oocyte complexes was not affected by CEBPα/β knockout and its mRNA fails to be down-regulated in CEBPα/β$^{gc-/-}$ cells in mice [4], we found that CEBPβ knockdown in porcine GCs could increase the expression of PTGS2 mRNA, implying that CEBPβ has a positive regulatory role on PTGS2. Furthermore, some down-regulated genes in CEBPα/β knockout mice [4], such as: PTGFR, Has2 and IGFBP4 in CEBPα/β$^{gc-/-}$ cells were also down-regulated in porcine GCs after knockdown of CEBPβ, which indicated that CEBPβ has similar regulatory roles in porcine GCs compared with mice. Considering that the expression of PTGS2 was increased along with decreased expression of IGFBP4, Has2 and PTGFR, we infer that CEBPβ may be involved in folliculogenesis, ovulation, and luteinization through regulation of these gene expressions in the porcine ovary.

It is well known that the cross talk between cell death and survival signals is very important for the follicular development and whether the follicle ultimately ovulates or undergoes degeneration is dependent on the multiple ovarian factors which regulate cell proliferation, differentiation and apoptosis. Previous studies have demonstrated that CEBPβ$^{-/-}$ cells fail to undergo apoptosis upon gamma IFN treatment, compared to wild-type control [34]. Similarly, CEBPβ induces cell death in human breast cancer cells [35]. Based on these studies, we predicted that CEBPβ knockdown might promote cell survival by lowering apoptosis in porcine GCs. In support of this, our results showed that CEBPβ knockdown could not induce apoptosis in porcine GCs. Moreover, we found an increase in mRNA level of bcl-2 and a decrease in mRNA expression of casp3 and p53 after CEBPβ down regulation. Caspase-3 has been reported as an important molecule in promoting apoptosis in all types of cells. Its activation leads to initiation of cascade of caspases, responsible for execution of cells [36]. Previous report has demonstrated that CEBPβ could activate the expression of p53, since its inactivation results in the loss of the apoptotic response [37]. The results in the present study showed that knockdown of CEBPβ led to the decrease in p53, indicating that it may inhibit porcine GCs apoptosis. However, knockdown of CEBPβ might not be able to further decrease the percentage of apoptosis (6.28 ± 0.20 % and 6.30 ± 0.77 % in CEBPβ knockdown group and control group, respectively) in this normal culture model of GCs. Whether CEBPβ knockdown could promote cell survival or inhibit apoptosis in porcine GCs, an induction model of apoptosis in porcine GCs has to be established in the future.

As CEBPβ has been involved in cell cycle regulation, therefore, we detected the cell cycle of GCs after CEBPβ knockdown. The result showed that S phase of cell cycle was significantly arrested compared to that of control (13.46 ± 3.22 % and 1.08 ± 0.71 %, respectively). Earlier reports showed that CEBPβ could inhibit cell proliferation in mouse fibroblast while proliferation is increased in its knockout model [38]. In the present study, GCs after CEBPβ knockdown showed decreased mRNA level of cyclin A1, which is a key regulator of the cell cycle progression from the S phase to the G2/M phase, resulted in the S phase arrest [39]. Whereas, cyclin D1 is a key regulatory protein that promotes the transition through the restriction point in the G1 phase to S phase [40]. Similarly, cyclin B1 is also an important regulator during the normal cell cycle progression [36], and its mRNA level was decreased after CEBPβ knockdown. Cyclin B1 inactivates Cdk1 after one cell cycle and therefore, allows mitotic exit and starting a new cell division [41]. Taken together, these results indicate that CEBPβ is important in normal GCs cell cycle control, growth and proliferation, particularly, just after ovulation when GCs are transformed into luteal tissue.

Furthermore, we used CEBPβ (346 amino acids in pig) antibody (sc-746) which could also react with and not be able to differentiate from CEBPα (354 amino acids in pig) by western blot. The results indicated that LH/hCG could significantly increase the expression of CEBPα/β protein in porcine GCs. Further results indicated that the mRNA level of CEBPβ could also be significantly increased by LH/hCG treatment. Although considering the compensational role of CEBPα to CEBPβ obtained in mouse model, CEBPβ$^{gc-/-}$ mice were subfertile, CEBPα$^{gc-/-}$ mice were fertile, and CEBPα/β$^{gc-/-}$ mice were completely infertile [4]; this results implied that CEBPα could compensate the roles of CEBPβ, but not necessary in some cases. CEBPβ is more important for fertility. In this study, we found that knockdown of CEBPβ alone in porcine GCs resulted in decreased estradiol and progesterone synthesis, and decreased gene expression (CYP11A1, IGFBP4, Has2 and PTGFR), which was consistent with the results in mice after knockout of CEBPα/β, indicating that CEBPβ alone is very important for porcine GCs function. However, there are some genes down-regulated (StAR and Runx2) or not affected (PTGS2) in mice model by knockout of CEBPα/β were up-regulated in porcine GCs after knockdown of CEBPβ, which

implied that CEBPβ might have different regulatory roles in porcine GCs compared with mouse model, or CEBPα might have a compensate function in some cases after knockdown CEBPβ.

In conclusion, this study revealed that CEBPβ might promote porcine GCs differentiate into lutein cells by regulation cell cycle and apoptosis, and increase estradiol and progesterone synthesis through controlling the expression of steroidogenic genes, which could promote folliculogenesis and ovulation. Its roles in the regulation of folliculogenesis, ovulation and luteal tissue formation were also evidenced by genes regulation by CEBPβ in the porcine GCs.

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