# Corticosterone stage-dependently inhibits progesterone production presumably via impeding the cAMP-StAR cascade in granulosa cells of chicken preovulatory follicles

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**ABSTRACT** Stress can suppress reproduction capacity in either wild or domestic animals, but the exact mechanism behind it, especially in terms of steroidogenesis, remains under-investigated so far. Considering the important roles of progesterone in avian breeding, we investigated the modulation of corticosterone on progesterone production in cultured granulosa cells of chicken follicles at different developmental stages. Using enzyme immunoassays, our study showed that corticosterone could only inhibit progesterone synthesis in granulosa cells from F5-6, F4, and F3 follicles, but not F2 and F1 follicles. Coincidentally, both quantitative real-time PCR and western blotting revealed that corticosterone could downregulate steroidogenic acute regulatory protein (**StAR**) expression, suggesting the importance of StAR in corticosterone-related actions. Using the dual-luciferase reporter system, we found that corticosterone can potentially enhance, rather than inhibit, the activity of *StAR* promoter. Of note, combining high-throughput transcriptomic analysis and quantitative real-time PCR, phosphodiesterase 10A (***PDE10A***), protein kinase cAMP-dependent type II regulatory subunit alpha (***PRKAR2A***) and cAMP responsive element modulator (***CREM***) were identified to exhibit the differential expression patterns consistent with cAMP blocking in granulosa cells from F5-6, F4, and F3, but not F2 and F1 follicles. Afterward, the expression profiles of these genes in granulosa cells of distinct developmental-stage follicles were examined by quantitative real-time PCR, in which all of them expressed correspondingly with progesterone levels of granulosa cells during development. Collectively, these findings indicate that corticosterone can stage-dependently inhibit progesterone production in granulosa cells of chicken preovulatory follicles, through impeding cAMP-induced StAR activity presumptively.

**Key words:** corticosterone, granulosa cells, progesterone, cAMP, StAR

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## INTRODUCTION

Steroidogenesis in the sex gland is an essential physiological process for reproduction, which regulates many breeding activities through steroid hormone actions (Johnson, 2011). Progesterone (**PG**) is one of the typical steroid hormones residing in the ovary and plays a crucial role in various reproductive processes, including ovulation, preparation of the uterus for implantation, and embryo development (Nakada et al., 1994; Diskin and Morris, 2008; Lonergan, 2011). In chicken, the granulosa cells (**GCs**) from hierarchical follicles (also termed preovulatory follicles) are the primary source of PG production, whereas the inner thecal layer produces androgens but limited amounts of PG (Johnson, 2011). Similar to other steroid hormones, the biosynthesis of PG is initiated with the transportation of cholesterol into mitochondria through steroidogenic acute regulatory protein (**StAR**) (Manna and Stocco, 2005). Thereafter, cytochrome P450 side chain cleavage enzyme (**CYP11A**) and hydroxysteroid dehydrogenase 3$\beta$ (**HSD3B**) completed the conversion from cholesterol to pregnenolone, and thereby PG, respectively (Storbeck et al., 2019). Normally, the PG synthesis is initiated by gonadotropins from the hypothalamic-pituitary origin, in which the gonadotropin receptor-mediated intracellular cyclic adenosine monophosphate



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(**cAMP**)/protein kinase A (**PKA**) singling can stimulate StAR, CYP11A, and HSD3B expression effectively (Johnson, 2011; Payne and Hales, 2004). Moreover, multifarious growth factors or cytokines locally secreted from ovarian cells were also proved to involve in the process via the autocrine and paracrine route (Johnson, 2015b; Manna and Stocco, 2011).

When animals get stressed, the hypothalamus-pituitary-adrenal (**HPA**) axis is activated to maintain homeostasis (Bu et al., 2019). As the terminal products of the HPA axis, the elevated glucocorticoids can regulate a broad spectrum of physiological processes through binding glucocorticoids receptor (**GR**) in target cells (Sapolsky et al., 2000). The link between stress and reproduction has been well-established previously, as evidenced by the substantial presence of gonadal dysfunctions when individuals are subjected to environmental stress or glucocorticoid exposure (Whirledge and Cidlowski, 2013). Moreover, steroid hormones originating from the sex gland were also proved to be involved in controlling the HPA axis (Bu et al., 2022). In chicken, both chronic and acute injections of glucocorticoids can result in the suppression of follicular development and laying performance (Williams et al., 1985; Shini et al., 2009). The expression of GR in the gonadal gland suggests the effects of glucocorticoids may be direct (Kwok et al., 2007), but the underlying mechanisms and molecular actions behind how this happens remain ill-identified currently. Moreover, the conflicting results make us more confused, because both promoting and inhibiting effects were reported in previous experiments (Whirledge and Cidlowski, 2013). In mice ovaries, glucocorticoid injection results in the apoptosis of both GCs and cultured cumulus cells via activating the Fas system (Yuan et al., 2016). However, studies conducted with bovine, rat and human GCs observed a protective role of glucocorticoids on serum deprivation or TNF-IFNG-induced apoptosis (Sasson and Amsterdam, 2003; Komiyama et al., 2008; Sasson et al., 2001). Similarly, glucocorticoids enhance gonadotropin-stimulated PG synthesis in rat and porcine GCs (Channing et al., 1976; Adashi et al., 1981), but inhibit gonadotropin-induced steroidogenesis in human and rat GCs (Hsueh and Erickson, 1978; Michael et al., 1993). Considering the apparent discrepancy of materials, drugs and doses in these studies, experiments conducted with appropriate models and the stress-driven dose may solve the doubts.

Recently, in cultured chicken follicle GCs, our study revealed that the stress-derived dose of corticosterone (**CORT**, the primary glucocorticoid in birds) can trigger antiproliferative and pro-apoptotic effects specifically in prehierarchical GCs, thereby confirming a direct etiology of ovarian disorders in response to stress perturbation (Yang et al., 2022a). However, the desensitization of hierarchical GCs to CORT-modulated proliferation and apoptosis still leads us to investigate some other functions in these cells, because the mRNA expression of GR in hierarchical GCs was equal to or even higher than that in prehierarchical GCs (Yang et al., 2022a). In chicken, the fundamental role of the hierarchical GCs is the production of PG (Johnson, 2011). Our preliminary data revealed that CORT can inhibit many transcripts responsible for steroid biosynthesis and steroid hormone biosynthesis in prehierarchical GCs. Together with the apparent inhibition of glucocorticoids on steroidogenesis of GCs in mammals (Hsueh and Erickson, 1978; Michael et al., 1993), these findings strongly emphasized that CORT may also inhibit PG production in hierarchical GCs, in a way to impede ovulation. Thus, using chicken as the model organism, the objectives of our present study are to evaluate the direct regulation and identify its relevant mechanism of stress-driven CORT on PG synthesis in GCs of hierarchical follicles at different developing stages.

## MATERIALS AND METHODS

### Animals and Tissues

Three to 400-day-old laying hens (Lohmann Layer strain) with normal follicular hierarchies were purchased from a local commercial company. For gene expression detection, 6 hens were sacrificed to collect GCs from 6 to 8 mm, F6, F5, F4, F3, F2, and F1 follicles respectively. The tissues were immediately frozen in liquid nitrogen until use.

### Chemicals, Reagents, Antibodies, and Primers

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. CORT was bought from Cayman Chemical (Ann Arbor, MI). Polyclonal anti-StAR antibody (A1035) and monoclonal antibody against $\beta$-actin (AC026) were purchased from ABclonal Technology (Wuhan, China). All primers used in this study were synthesized by Tsingke Biological Technology Co., Ltd. (Chengdu, China) and listed in Table 1. All the PCR products were confirmed by sequencing to ensure the specificity of the used primers.

### Cell Culture and Drug Treatment

According to our previously established method (Yang et al., 2022a), GCs from 6 to 8 mm, F5-6, F4, F3, F2, and F1 follicles were isolated from one chicken ovary and digested by type I collagenase (Yuanye Bio-Technology Co., Ltd, Shanghai, China) at 37°C for 20 min, respectively. The dispersed cells were filtered through 200 mesh cell sieves and then diluted to a density of approximately $5 \times 10^5$ cells/mL. Cells were then plated into a 48-well plate (NEST Biotech, Wuxi, China) in 200 $\mu$L of culture medium containing 10% (vol/vol) fetal bovine serum (Gibco) and cultured at 37°C with 5% $CO_2$. Cells were cultured overnight before the medium was removed. Cells were then treated by DMEM/F12 medium containing 2.5% (vol/vol) fetal bovine serum (**FBS**) with or without CORT in stress derived dose (100 nM) for 24 h. All animal experimental protocols

**Table 1.** Primers used in the present study.

| Primer name | Sense/antisense | Primer sequence (5'- to- 3') | Size (bp) |
|---|---|---|---|
| *Primers for quantitative real-time PCR* | | | |
| LSS-qF1 | Sense | GTTATGCCAAGCGTCTGTCA | 220 |
| LSS-qR1 | Antisense | ATGGCTCGCTGACGTAGGTA | |
| SQLE-qF1 | Sense | GAGAATGTGTCTCAGGTCCT | 133 |
| SQLE-qR1 | Antisense | ATGCTGATCCACGACTCCGA | |
| CYP51A1-qF1 | Sense | GGAGAAGTTCGCCTACATTC | 171 |
| CYP51A1-qR1 | Antisense | GGCGTATGTATCATGGTTGT | |
| EBP-qF1 | Sense | CATGACGAGCGATGACTTCA | 172 |
| EBP-qR1 | Antisense | CGTGGCGAAGTAGAGGATGT | |
| DHCR24-qF1 | Sense | GAGCTTGATGACCTCACTGT | 107 |
| DHCR24-qR1 | Antisense | ACCAGTTCGTAGGCCATGCA | |
| FDFT1-qF1 | Sense | GACTATCTAGAGGACCAGCT | 243 |
| FDFT1-qR1 | Antisense | AGTGGCAATAGCCATCACCT | |
| MSMO1-qF1 | Sense | CTGCATCGACTGCTGCATCA | 127 |
| MSMO1-qR1 | Antisense | CAGCTCCAAGGATGAGCGTT | |
| DHCR7-qF1 | Sense | TACGGCTGCTGGAATCTATG | 126 |
| DHCR7-qR1 | Antisense | GGAGTGACAGCACCTTCTTG | |
| StAR-qF1 | Sense | CACTGCACCGCAGAGATGCT | 134 |
| StAR-qR1 | Antisense | ACGACAGCTTGCTGAGCTCCT | |
| CYP11A1-qF1 | Sense | TCCGCTTTGCCTTGGAGTCTGTG | 112 |
| CYP11A1-qR1 | Antisense | ATGAGGGTGACGGCGTCGATGAA | |
| HSD3B1-qF1 | Sense | GATGAGGCGCTGGCTGAGAT | 145 |
| HSD3B1-qR1 | Antisense | GACAGGCACGGTGCAGGAAT | |
| PDE8A-qF1 | Sense | TGTGGGTCCAGAGAATGTCTA | 87 |
| PDE8A-qR1 | Antisense | GTCCCAAGCAGAGCTCCATC | |
| PDE10A-qF1 | Sense | AGAGATTGTCTGATGCTCAAAAC | 200 |
| PDE10A-qR1 | Antisense | GTGTGCCTTTGCTGGATTGG | |
| PKAR2A-qF1 | Sense | ATCGTGTTCTGTGCGATCTTG | 171 |
| PKAR2A-qR1 | Antisense | TCACAACCAGGCACTTTGCT | |
| CREM-qF1 | Sense | GAATTTCTCCACTGTCCATGC | 157 |
| CREM-qR1 | Antisense | TCCTTCTCAGCAAGTCATCTCT | |
| ATF6-qF1 | Sense | CGTCGTCTGAACCACTTACTGA | 101 |
| ATF6-qR1 | Antisense | CCTTCTTTCCTAACAGCCACAC | |
| $\beta$-actin-qF1 | Sense | CACCATTGGCAATGAGAGGT | 123 |
| $\beta$-actin-qR1 | Antisense | CAGAGTACTTGCGCTCAGGT | |
| *Primers for constructing pGL3-STARp reporter vector* | | | |
| StAR-pF1 | Sense | CGGGGTACCGCAGTGCCCGTGTTATCTTA | 1533 |
| StAR-pR1 | Antisense | CCGCTCGAGGCTTGTCAGCACCGAGATAA | |
| GR-F1 | Sense | CGCGGATCCGAGCTGATGTTAAAATCATGGA | 2560 |
| GR-R1 | Antisense | CCGGAATTCCGCTGCTTCTATAGCCTA | |

Restriction sites added in 5′-end of the primers are underlined.

employed in this study were approved by the Animal Ethics Committee of Sichuan Agricultural University.

## Total RNA Extraction, RT-PCR and Quantitative Real-time PCR

Based on the manufacturer's instructions and our previously established method (Yang et al., 2022b), total RNA was extracted from either tissues or primary cultured GCs by RNAzol (Molecular Research Center, Cincinnati, OH) and dissolved in diethylpyrocarbonate (**DEPC**)-treated $H_2O$. These RNA samples were then used for reverse transcription (**RT**) based on Moloney murine leukemia virus (**MMLV**) reverse transcriptase (Takara, Dalian, China). Briefly, oligodeoxythymide and total RNA (1 $\mu$g) were mixed in a total volume of 5 $\mu$L, incubated at 70°C for 10 min, and cooled at 4°C for 2 min. Then, the first strand buffer (containing 0.5 mM of each deoxynucleotide triphosphate and 100U reverse transcriptase) was added to the reaction mix at a total volume of 10 $\mu$L. RT was performed at 42°C for 90 min. RT-negative controls were performed under the same condition without the addition of reverse transcriptase.

Quantitative real-time PCR (**qRT-PCR**) was performed according to the previously established method (Yang et al., 2020). Briefly, the PCR reaction was carried out at 94°C of denaturation for 2 min, followed by 40 reaction cycles (94°C for 20 s, 60°C for 20 s and 72°C for 20 s) and the fluorescence signal was detected at 72° C. The mRNA levels of genes were first calculated as the ratio to that of $\beta$-actin and then expressed as the fold difference compared with either the control or 6 to 8 mm group. To confirm the specificity of PCR amplification, melting curve analysis, agarose gel electrophoresis plus sequencing were performed at the end of the reaction.

## Progesterone Enzyme Immunoassays

The medium of cultured GCs was collected immediately after CORT treatment for 24 h. A commercial enzyme-linked immunosorbent assay (**ELISA**) kit (Catalog no. E-EL-0154c, Elabscience, Wuhan, China) was performed to quantify the PG levels in medium samples according to the manufacturer's instructions. The absorbance at 450 nm was measured by a microplate reader (PerkinElmer, Victor X3, Germany) and the

standard curve was fitted using Origin 9 (Northampton, MA).

## Western Blotting

Based on our previously established method (Bu et al., 2016), western blotting was employed to detect the protein levels of StAR in cultured GCs. The cell lysates were resolved by electrophoresis in a 15% SDS-PAGE and the bands from western blotting were quantitated by densitometric analyses (Image J software, NIH). The relative StAR levels were calculated as the ratio to that of $\beta$-actin and then expressed as the percentage of the control group (without CORT treatment).

## Plasmid Construction

According to the genomic sequence of chicken *StAR*, gene-specific primers containing restriction enzymes cut sites (*Kpn* I and *Xho* I) were designed and used to amplify its 5'-flanking regions using high-fidelity Taq DNA polymerase (TOYOBO, Osaka, Japan). Using the predicted sequence of chicken *GR* (XM_046927040.1) deposited in GenBank as references, specific primers containing restriction enzymes cut sites (*BamH* I and *EcoR* I) were also designed to amplify the ORF of *GR* from GCs. The amplified two PCR products were then digested by corresponding enzymes (TransGen Biotech, Beijing, China) and inserted into the pGL3-Basic and pcDNA3.1 vector respectively. Restriction enzyme double digestion, agarose gel electrophoresis and sequencing were performed to ensure the correctness of constructed plasmids.

## Luciferase Reporter Assays

Human embryonic kidney epithelial (**HEK293T**) cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% (vol/vol) FBS, 100 U/mL of penicillin G and 100 $\mu$g/mL of streptomycin (HyClone, Logan, UT) in a 90-mm dish (Corning, NY) and incubated at 37°C with 5% $CO_2$. As described previously (Bu et al., 2013), the promoter activities of pGL3-StARp were determined in cultured HEK293T cells using the dual-luciferase reporter assay (Promega,

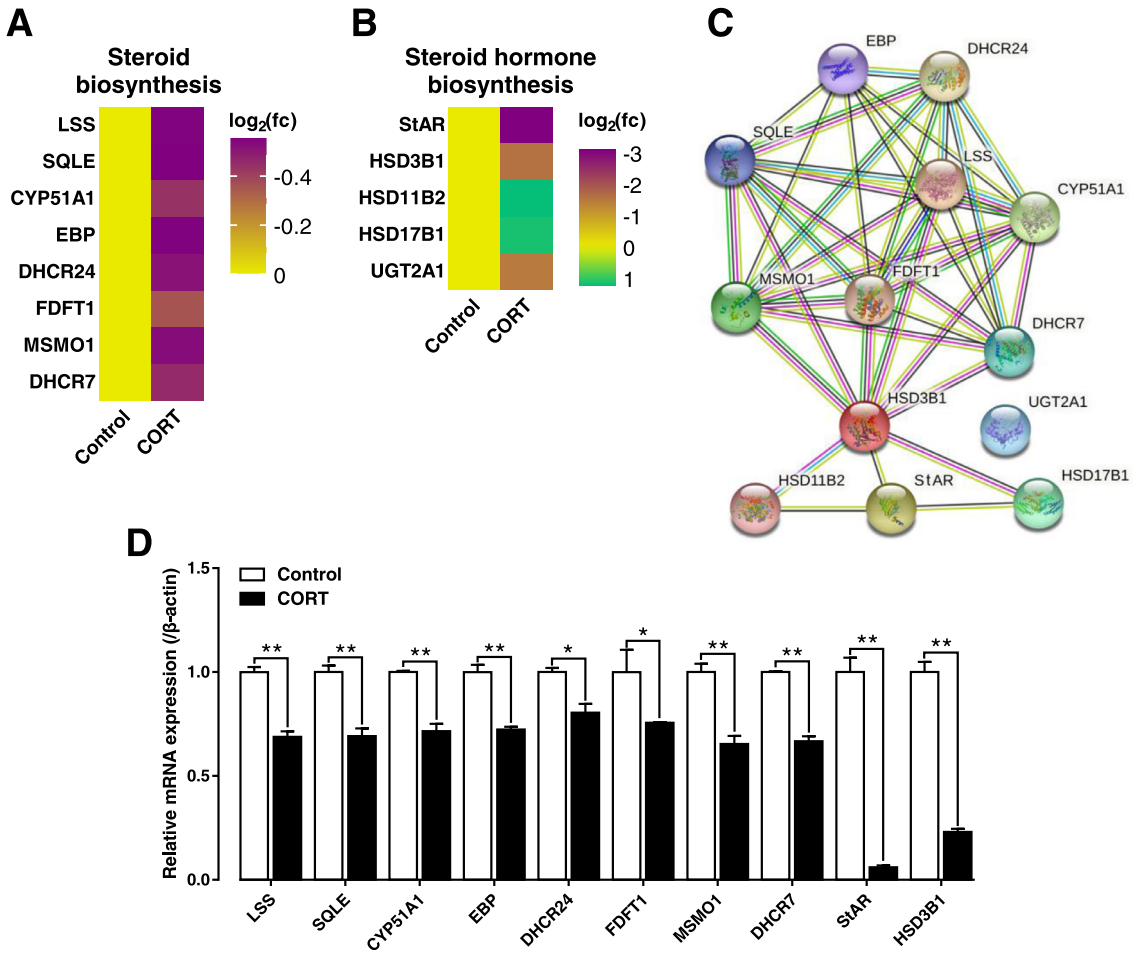


**Figure 1.** KEGG analyses of DEGs between control and CORT treated group in 6−8 mm GCs. (A-B) Heat map shows the DEGs enriched into the steroid biosynthesis (A) and steroid hormone biosynthesis (B) signaling pathway, respectively. (C) The protein-protein interaction between the above enriched DEGs is based on the STRING database. (D) qRT-PCR validation of several DEGs identified in transcriptome sequencing, including *LSS, SQLE, CYP51A1, EBP, DHCR24, FDFT1, MSMO1, DHCR7, StAR*, and *HSD3B1*. Each data point represents the mean ± SEM of at least three replicates (N ≥ 3). Significant differences between CORT treatment and control group are indicated with asterisks * or **, reflecting $P < 0.05$, $P < 0.01$, respectively.

Madison, MI). To evaluate the direct effect of CORT on StAR transcription, cells were co-transfected with pcDNA3.1-GR and pGL3-StARp and treated with or without 100 nM CORT. The roles of cAMP on StAR expression were also investigated in pGL3-StARp transfected cells treated with or without 1$\mu$M forskolin (adenylate cyclase agonist). Luciferase activity in cells was normalized to renilla luciferase activity derived from the pRL-TK vector and then expressed as a relative fold increase compared with the control group.

### Data Analysis

Statistical analysis based on the collected data was conducted in GraphPad Prism 7 (GraphPad Software, San Diego, CA), and the mean of replicates (N $\geq$ 3) was evaluated to be displayed as mean $\pm$ SEM. Significance between different groups was determined using the unpaired t-test and presented as $P < 0.05$ ($*$) or $P < 0.01$ ($**$). To validate our results, all in vitro experiments were repeated at least 3 times, and representative data are reported. Each repeated experiment was performed by using primary cultured granulosa cells from different individuals.

## RESULTS

### Effect of CORT on PG Production in Chicken Ovarian Hierarchical GCs

In our recent study, a high-throughput transcriptomic analysis conducted with 6 to 8 mm GCs (Accession number: CRA006114, https://ngdc.cncb.ac.cn/gsa/.) has identified 1362 differentially expressed genes (**DEGs**) (Yang et al., 2022a). By using the Kyoto Encyclopedia of Genes and Genomes pathway analysis, we found that eight DEGs have enriched in steroid biosynthesis (gga00100), while 5 DEGs have enriched in steroid hormone biosynthesis (gga00140) signaling pathway (Figure 1A, 1B and S1). Moreover, protein-protein interaction analysis based on the STRING database showed dense and complex connections among these genes (Figure 1C), suggesting their importance in CORT actions in GCs. qRT-PCR analysis was then performed to confirm the gene expression profiles obtained from high-throughput RNA sequencing. As shown in Figure 1D, the mRNA levels of all identified genes (*LSS, SQLE, CYP51A1, EBP, DHCR24, FDFT1, MSMO1, DHCR7, StAR, HSD3B1*) showed a significant decrease in primary cultured GCs after CORT treatment, which were in line with the high-throughput sequencing data. Considering the downstream role of HSD11B2, HSD17B1, and UGT2A1 in PG production, these genes were not detected in the present study.

Because PG is the predominant steroid hormone produced by GCs in chicken, we further utilized the ELISA assay to detect the progesterone levels in hierarchical follicles with different sizes (F5-6, F4, F3, F2, F1) after CORT treatment (Figure 2A). The results showed that

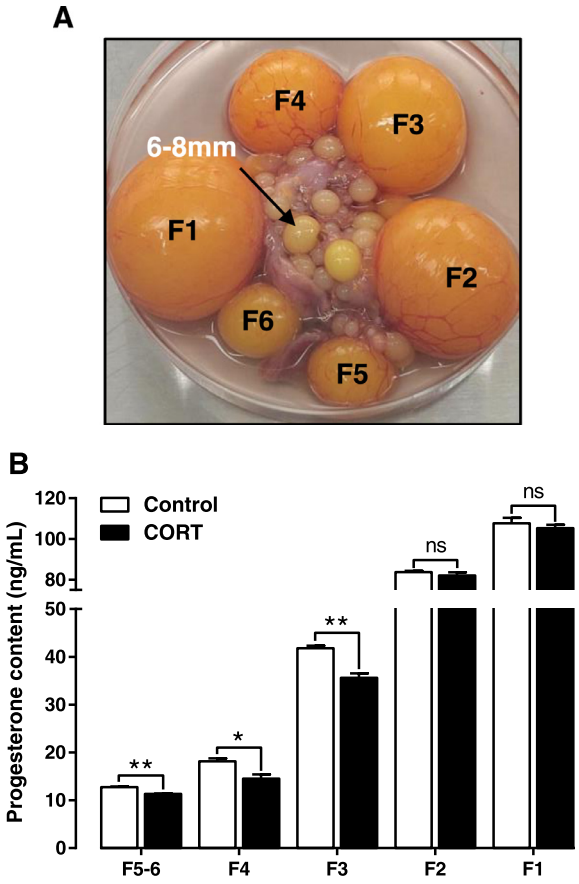


**Figure 2.** (A) The illustration of the chicken ovary during the laying period. 6−8 mm prehierarchical follicles were labeled by a black arrow, whereas 6 large preovulatory follicles (F1−F6) were marked on the surface. F1 presents the largest follicle. (B) ELISA detection of PG levels (ng/mL) in GCs from F5-6, F4, F3, F2, and F1 follicles. Each data point represents the mean $\pm$ SEM of at least three replicates (N $\geq$ 3). Significant differences between CORT treatment and control group are indicated with asterisks * or **, reflecting $P < 0.05$, $P < 0.01$, respectively. ns, nonsignificant.

100 nM of CORT could only reduce the progesterone production in GCs from F5-6, F4, and F3 follicles, while portraying a nonsignificant role in GCs from F2 and F1 GCs (Figure 2B).

### Effect of CORT on the Expression of Key Genes Related to PG Biosynthesis

To elucidate the relevant mechanism behind how CORT influences PG production in GCs from hierarchical follicles, qRT-PCR analysis was carried out to detect the expression profiles of several key genes involved in PG production, including *StAR, CYP11A1* and *HSD3B1*. The results showed that 24 h of CORT treatment failed to trigger any expression change of *CYP11A1* (Figure 3B) and *HSD3B1* (Figure 3C) in GCs from all sizes of hierarchical follicles. Notably, CORT could down-regulated the mRNA expression of *StAR* in GCs from F5-6, F4, and F3 follicles, but not GCs from F1 and F2 follicles (Figure 3A), suggesting the correlation between *StAR* expression and CORT blocked PG production. We also detected the relative changes of StAR protein in response to CORT by using western

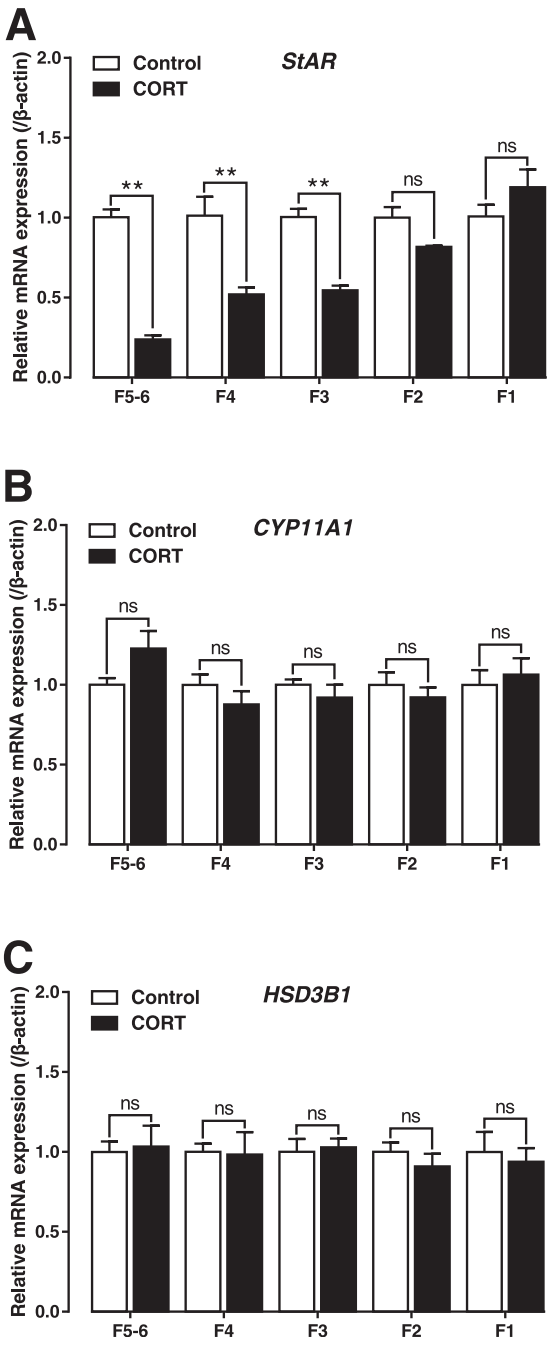


**Figure 3.** (A-C) qRT-PCR detection of mRNA expression of *StAR* (A), *CYP11A1* (B) and *HSD3B1* (C) in GCs from F5-6, F4, F3, F2, and F1 follicles in response to CORT. Each data point represents the mean ± SEM of at least three replicates (N ≥ 3). $^{*}P < 0.05$. $^{**}P < 0.01$. ns, nonsignificant.

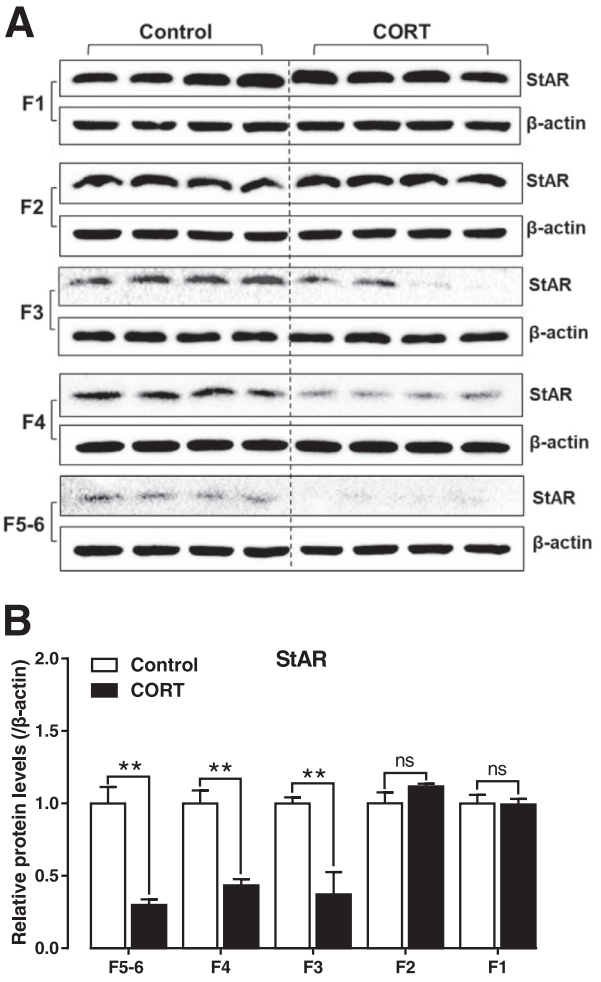


**Figure 4.** (A) Western blotting shows the protein levels of StAR in cultured GCs from F5-6, F4, F3, F2, and F1 follicles in response to CORT. (E) Their relative levels were normalized by that of $\beta$-actin in GCs lysate and then expressed as a fold change compared to the control group. Each data point represents the mean ± SEM of 4 replicates (N = 4). $^{**}P < 0.01$ vs. control treatment group. ns, nonsignificant.

blotting. Similarly, CORT administration significantly decreased the protein accumulation of StAR in F5-6, F4, and F3 GCs, while having an insignificant influence on F2 and F1 GCs (Figure 4A, 4B).

## Identification of the Direct Regulation of CORT on StAR Transcription

Based on the predicted glucocorticoid response elements (**GREs**) located upstream from the transcription initiation site of the StAR gene (Figure S2), we further constructed the pGL3-StARp (-1615/-101 Luc) vector to identify whether CORT could directly regulate StAR expression by using a dual-luciferase reporter assay (Figure 5A). As shown in Figure 5B, transfected with pGL3-StARp in HEK293T cells exhibited an enhanced luciferase activity relative to that of the pGL3-Basic group, suggesting that our cloned region possessed strong promoter activity. Thereafter, pcDNA3.1-GR and pGL3-StARp were co-transfected into HEK293T cells and treated with or without CORT (Figure 5C). The results showed that CORT administration significantly enhanced the luciferase activity of cells, indicating that the CORT-GR pair could directly initiate *StAR* transcription via targeting the *StAR* promoter.

According to the predicted numerous cAMP responsive element modulator (**CREM**) on the *StAR* promoter region (Figure S2), we also detected whether cAMP activating could stimulate *StAR* expression in the present study. As expected, forskolin in a dose of 1$\mu$M significantly up-regulated the luciferase activity of HEK293T cells transfecting pGL3-StARp (Figure 5D).

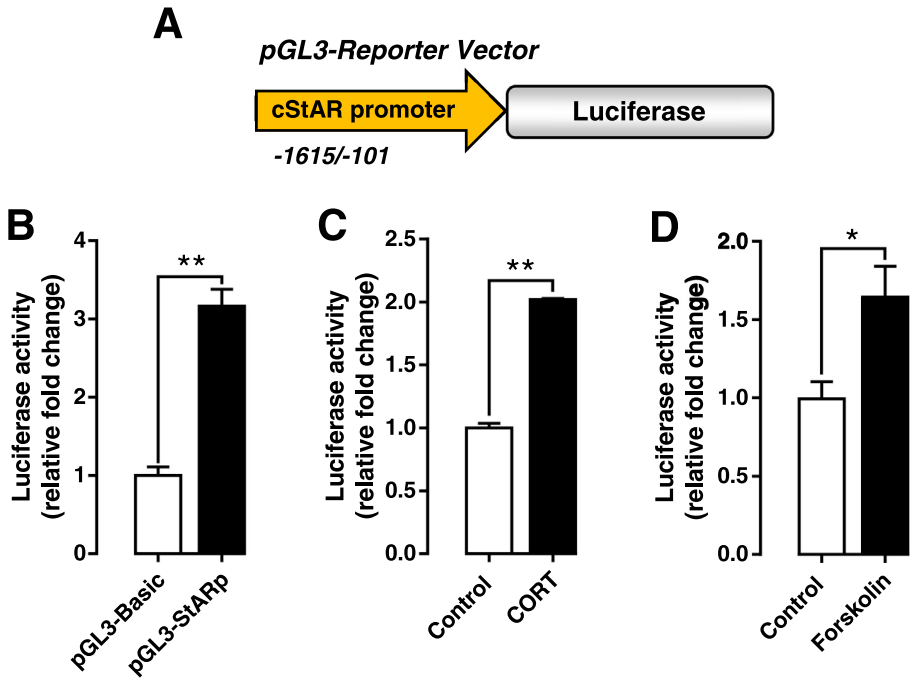


**Figure 5.** (A) The illustration of the pGL3-StARp-Luc reporter vector constructed in the present study. The 5'-flanking region (−1615 to −101) upstream of chicken *StAR* was cloned into a pGL3-Basic vector for the generation of promoter-luciferase constructs. (B) This promoter-luciferase construct (or empty pGL3-Basic vector) was then co-transfected into HEK239t cells along with the pRL-TK vector and its promoter activities were determined by the Dual-luciferase reporter assay. (C) Effect of CORT on the luciferase activity of HEK239t cells co-transfected with pGL3-StARp and pcDNA3.1-GR. (D) Effect of forskolin (2 $\mu$M) on the luciferase activity of HEK239t cells transfecting pGL3-StARp vector. Each value represents the mean ± SEM of at least three replicates (N ≥ 3). $^{*}P < 0.05$. $^{**}P < 0.01$. ns, nonsignificant.

### *Effect of CORT on the Expression of Genes Related to cAMP Signaling*

Given the integral role of cAMP signaling in *StAR* expression and PG production, a high-throughput sequencing analysis coupled with qRT-PCR assay was conducted to investigate whether CORT could inhibit *StAR* expression in a cAMP-dependent manner. Based on the high-throughput sequencing data, we have identified eight DEGs that showed close interaction at their protein levels and related to the cAMP route (Figure 6A, B, and S3). The bio-significance of these DEGs was then assessed according to the GeneCards database and found that five of them showed coincident expression changes with cAMP blocking after CORT treatment, including *CREM, ATF6, PDE8A, PDE10A,* and *PRKAR2A* (Figure 6A). We next performed qRT-PCR to determine the expression changes of the above five genes in F5-6, F4, F3, F2, and F1 GCs in response to CORT. As shown in Figure 6C, 24 h administration of CORT triggers diverse expression profiles of these genes in all detected GCs. Of note, *PDE10A, PRKAR2A,* and *CREM* were the genes altered consistently with CORT-inhibited StAR expression and PG production events, suggesting their predominant roles in these processes. In addition, the consistent differential expression patterns of these genes in 6 to 8 mm GCs with that of transcriptome analysis once again confirmed the reliability of RNA-seq data.

### *Expression Profile of StAR, PDE10A, PRKAR2A, and CREM in Developing Ovarian GCs*

To elucidate the potential roles of StAR, PDE10A, PRKAR2A, and CREM in chicken ovarian, we further took a qRT-PCR approach to detect their expression profile in GCs at different development-stage follicles in the chicken ovary. As shown in Figure 7A and C, the mRNA levels of *StAR* and *PRKAR2A* showed an uninterrupted increase through follicular growth. In contrast, the mRNA expression of *PDE10A* processed a remarkable increase after follicular selection (from 6 to 8 mm to F6 GCs), but then constantly diminished in the remaining stages before ovulation (Figure 7B). Unlike the above genes revealing an increase or decrease trend, the expression profile of *CREM* remains immutable in all hierarchical GCs, which are generally higher than that of 6-8mm GCs (Figure 7D).

## DISCUSSION

The GCs of ovarian hierarchical follicles can produce prodigious amounts of PG, a steroid hormone critical for follicular development and ovulation in chicken. The present study provides new insight into the exact actions and potential mechanism of stress-driven CORT in controlling GCs steroidogenesis. In primary cultured GCs of chicken hierarchical follicles, the studies showed that

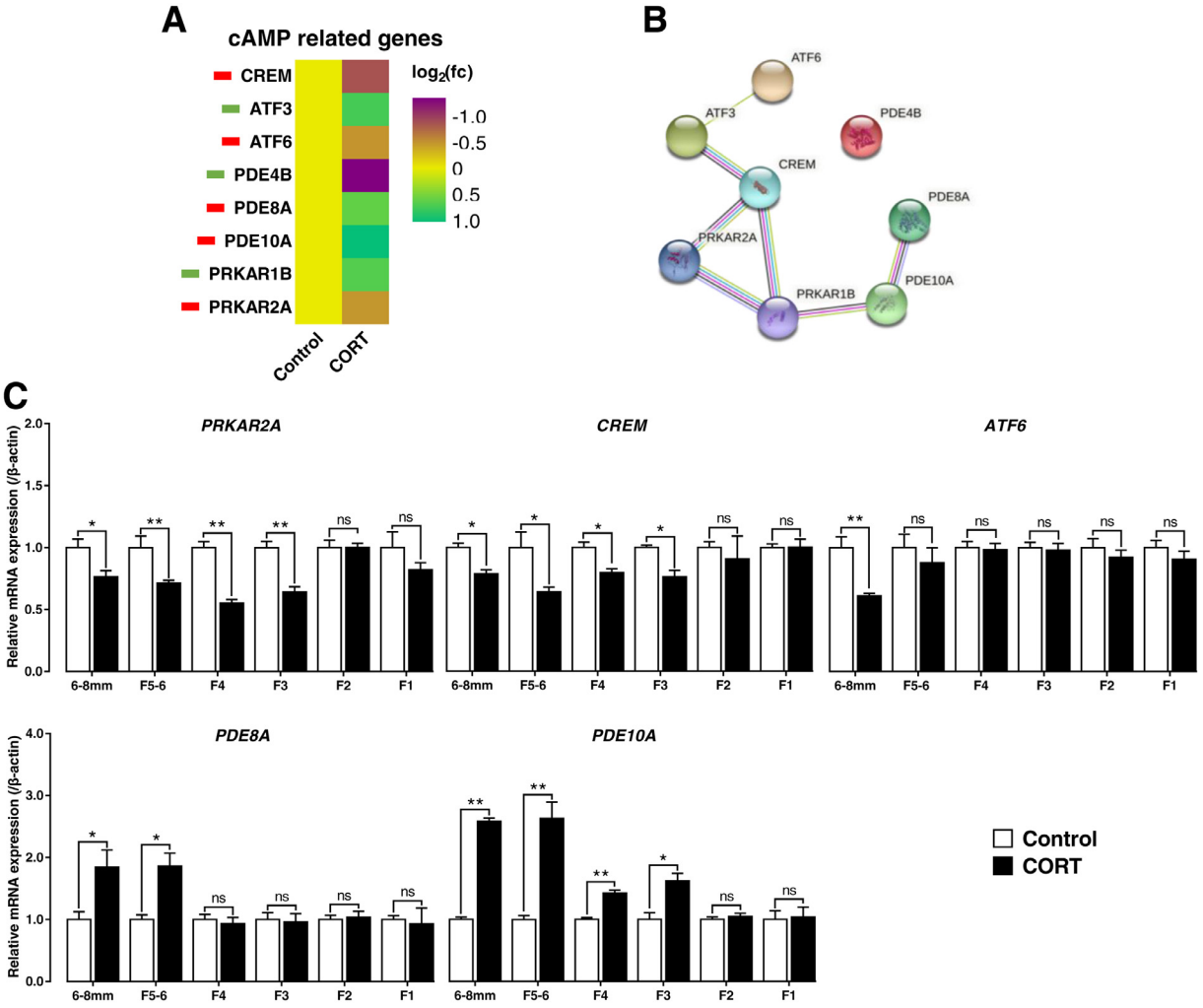


**Figure 6.** (A) The heat map shows the DEGs related to cAMP signaling in 6−8 mm GCs suffering CORT treatment. The red rectangle represents genes consistent with cAMP blocking, while the green represents the opposite trend. (B) The protein-protein interaction of these DEGs is based on the STRING database. (C) qRT-PCR detection of mRNA expression of *PRKAR2A, CREM, ATF6, PDB8A*, and *PDB10A* in GCs from 6 to 8 mm, F5-6, F4, F3, F2, and F1 follicles in response to CORT. Each data point represents the mean ± SEM of at least three replicates (N ≥ 3). $^{**}P < 0.05$. $^{**}P < 0.01$. ns, nonsignificant.

CORT can only inhibit PG production in GCs from F5-6, F4, and F3 follicles, but not F2 and F1 follicles. qRT-PCR and western blot revealed that the stage-dependent effect was consistent with the expression changes of StAR. Dual-luciferase reporter assays coupled with qRT-PCR further demonstrated that CORT might block StAR expression through impeding cAMP signaling, rather than in a direct manner mediated by nuclear GR. Moreover, the expression profile of *StAR* and key cAMP-related factors were also evaluated in chicken ovarian GCs at different development-stage. To our knowledge, our study presents the first to investigate the direct action and relevant mechanism of glucocorticoid in modulating steroidogenesis of follicle GCs in avian species including chicken.

The ability of glucocorticoids in regulating steroidogenesis in ovarian GCs has been amply documented in mammals (Geraghty and Kaufer, 2015; Wei et al., 2019), but a similar process presides over avian species have not been reported so far. Given the significance of PG in avian ovulation, it is proposed that stress-driven glucocorticoids impede reproductive ability, at least in part, by blocking the steroidogenesis of GCs. This perspective was then supported by our preliminary data, as high-throughput RNA sequencing analysis together with qRT-PCR revealed that CORT treatment in primary cultured 6-8mm GCs evoked transcriptional inhibition of the enzymes involved in steroidogenesis. Considering slow-growing 6-8mm follicles can only produce no measurable PG, we next investigated the direct actions of CORT on steroidogenesis of GCs from hierarchical follicles. In chicken, the PG production was initiated immediately subsequent to the follicular selection, which arrived at its peak in the largest F1 follicle (Johnson, 2011). In accordance with that, the basal PG levels secreted by cultured ovarian GCs enhanced continuously with follicular development, suggesting the reliability of our established in vitro models. Indeed, our results showed that CORT treatment significantly down-regulated the PG levels in cultured GCs, thereby confirming the above presumption. Intriguingly, that kind of modulation could only be processed in F5-6, F4, and F3 GCs, whereas the GCs from large F2 and F1 follicles depicted negligible influence after CORT

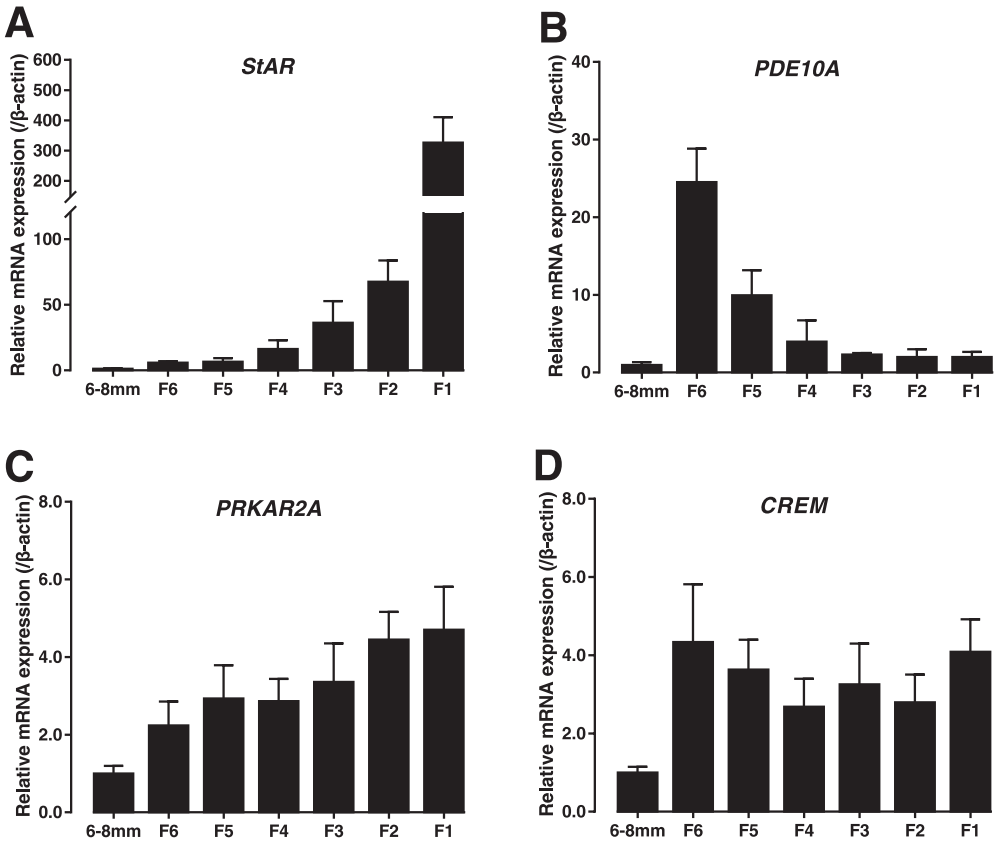


**Figure 7.** (A) qRT-PCR detection of *StAR, PDB10A, PRKAR2A*, and *CREM* in chicken ovarian GCs from follicles at different development-stage (including 6−8 mm, F5, F6, F4, F3, F2, and F1). The measured samples are collected from 6 individuals (N = 6).

treatment. As mentioned previously, using the same experimental models, our group has proved that CORT could only trigger proliferative and apoptotic effects in 6-8mm prehierarchical GCs, this stage-dependent regulation may also indicate a delicate balance between promotion and suppression signals, which is in keeping with the physiological features of GCs during development (Yang et al., 2022a). GCs in newly recruited small hierarchical follicles showed high sensitivity in repose to CORT, possibly due to the low-level growth-promoting signaling at the early stage (Johnson, 2015a). The PG inhibition induced by CORT might be gradually attenuated by yolk deposition through follicular development, as a previous in vivo study proved that sufficient energy feeding could eliminate CORT exposure triggered ovarian dysfunction and laying suppression in chicken (Wang et al., 2013). Moreover, CORT-induced transcriptional inhibition of steroidogenesis related genes in 6-8mm GCs might also be related to its role in GC differentiation and follicular selection, which are worth for further study.

The biosynthesis of steroid hormone is highly conserved among vertebrate species, which involves a canonical StAR-P450scc enzymatic cascade using cholesterol as substrate (Storbeck et al., 2019). Despite the terminal effects and defining roles of CYP11A1 and HSD3B within PG synthesis, our study conducted with qRT-PCR and western blotting only illustrated the inhibition of StAR expression in GCs upon CORT treatment, whereas the mRNA levels of both *CYP11A1* and *HSD3B* remain unchanged in GCs of all preovulatory follicles. Of particular note, the expression blocking of StAR in both mRNA and protein levels appeared as a stage-dependent profile, which was in line with the CORT-induced inhibitory effects on PG synthesis. These findings herein support the possibility that CORT evokes steroidogenesis inhibition via blocking STAR expression primarily. The importance of StAR activity has been well-documented previously, according to its highly homologous structure and rate-limiting role in steroidogenesis (Bauer et al., 2000). StAR is responsible for the transportation of cholesterol to the site of P450scc within the inner mitochondrial membrane, and is commonly regarded as the prerequisite for the synthesis of steroid hormones (Jefcoate, 2002; Manna and Stocco, 2005). In consist with the origination of PG synthesis, the expression of *StAR* in chicken was also initiated after follicular recruitment (Bauer et al., 2000). The enhanced capacity for PG production is tightly related to the particularly abundant expression of StAR, specifically within the F1 follicle GCs (Johnson and Bridgham, 2001). Coincidentally, our qRT-PCR analysis revealed that the expression of *StAR* in GCs was elevated constantly with development, further supporting the link between StAR and PG synthesis. Taken together, these findings strongly emphasized that StAR could serve as a potential biomarker of follicular selection and PG production in chicken. Despite all

these, further experiments regarding the overexpression and knockdown are still required to elucidate its exact role within CORT evoked inhibition of steroidogenesis.

The biological actions of glucocorticoids are primarily initiated through the interaction with GR, which served as a transcription factor to regulate mRNA levels of a broad spectrum of transcripts (Weikum et al., 2017). Since many GREs were predicted to be located in the promoter region of the *StAR* gene, it is proposed that the CORT-GR complex can regulate *StAR* expression via targeting the *StAR* promoter directly. However, our dual-luciferase assay revealed that CORT significantly enhanced the luciferase activity of HEK293t cells transfecting *StAR* promoter and *GR*, suggesting that CORT-GR is capable of directly stimulating, rather than suppressing, *StAR* expression. This result is contrasted to the above findings revealed by qRT-PCR and western blotting, since it was found that the CORT stage-dependently inhibits StAR expression in hierarchical GCs. Thus, some mechanisms aside from transcriptional modulation might be involved in the CORT-induced suppression of StAR expression in cultured chicken ovarian GCs. In addition to the principal effects of glucocorticoids in transcriptional modulation, these hormones are also capable of eliciting rapid cellular responses through nongenomic mechanisms (Oakley and Cidlowski, 2013). In rat aortic smooth muscle cells, dexamethasone can inhibit vasoactive agents or cAMP activators induced cAMP accumulation through a dose-dependent way (Ito et al., 1994). Moreover, the activity of various kinases, such as phosphoinositide 3-kinase and mitogen-activated protein kinases, could also be impinged by glucocorticoids (Oakley and Cidlowski, 2013). Collectively, these findings lead to a possibility that stress-driven CORT may suppress StAR expression in an indirect manner, including regulating intracellular signaling nodes presumptively.

In chicken, the intracellular initiation of both StAR and P450scc protein is dependent upon cAMP signaling (Johnson, 2011). In particular, StAR represents an early response gene that can be significantly increased within three hours by elevated intracellular cAMP levels (Balasubramanian et al., 1997; Johnson and Bridgham, 2001). Our experiment conducted with HEK293T cells revealed that forskolin treatment remarkably enhanced the luciferase activity of cells transfecting pGL3-StARp, further validating the positive regulation of cAMP among *StAR* transcription. The involvement of CORT in hindering cAMP signaling transduction has been partly supported in the present study, as evidenced by the identification of eight DEGs involved in cAMP signaling in 6-8mm GCs. Significantly, five DEGs of them showed consistent expression changes with cAMP blocking. The expression changes of the above five genes in different-stage hierarchical GCs in response to CORT were then investigated by qRT-PCR, and found that *CREM, PDE10A*, and *PRKAR2A* were the transcripts shown coincident change profiles with CORT-induced stage-dependent effects. As described previously, CREM and PRKAR2A (genes encoding PKA) are two critical factors transducing cAMP signaling (Manna et al., 2002; Payne and Hales, 2004), whereas PDE10A (phosphodiesterase 10A) could serve as a hydrolase using cAMP as substrate (Nishi et al., 2008). The accumulation of *PDB10A* plus the decrease of *PRKAR2A* and *CREM* transcripts strongly emphasizes that CORT can impede cAMP signaling in hierarchical GCs. Because of the scant collection of cAMP in our cultured GCs, we failed to evaluate the relative changes in cAMP levels in the present study. However, together with the previous study showing the antagonistic role of dexamethasone in cAMP-induced *STAR* transcription in mouse Leydig cells (Martin and Tremblay, 2008), a study presented here still raises a possibility that CORT inhibits *STAR* expression in a cAMP-dependent manner. Further studies regarding cAMP detection and chromatin immunoprecipitation are required to elucidate the mechanism more exquisitely.

In this study, we also detected the expression profile of *PDE10A, PRKAR2A*, and *CREM* in different developmental-stage GCs using qRT-PCR. As mentioned above, both StAR expression and PG production ability were initiated in the hierarchy stage, and enhanced gradually with follicular growth (Johnson, 2011). Accordingly, the expression profile of *PRKAR2A* in GCs also showed a continuously increasing trend through development. Furthermore, the mRNA levels of cAMP hydrolase (*PDE10A*) were gradually diminished in hierarchical GCs close to ovulation, suggesting their importance between StAR expression and PG synthesis in vivo. Interestingly, we have observed a remarkable increase in *PDE10A* levels before and after follicular selection. Considering the negligible level of cAMP in prehierarchical GCs (Johnson, 2015a), it is not surprising why *PDE10A* showed scant expression in this stage. However, understanding the exact mechanism behind how *PDE10A* is evoked during follicular recruitment might be an interesting question, as it will help to illustrate the dynamic regulation during follicular recruitment. Different from the expression pattern of *PRKAR2A* and *PDE10A*, the mRNA levels of *CREM* remain relatively unchanged in all hierarchical GCs detected, but are more abundant than that in prehierarchical GCs. These findings taken together, further supported the significance of *PRKAR2A, PDE10A*, and *CREM* in CORT-induced ovarian inhibition, especially StAR expression and PG production.

In summary, the results showed that the stress-driven dosage of CORT could stage-dependently inhibit PG production in the GCs of chicken preovulatory follicles, thus suppressing follicular development and ovulation potentially. qRT-PCR and western blotting revealed that these effects might be implemented via inhibiting StAR expression. Dual-luciferase reporter assays coupled with qRT-PCR further demonstrated that cAMP signaling, especially PDE10A, PKA, and CREM nodes, might play important roles in CORT-blocked StAR expression (Figure 8). Collectively, these results indicate the direct and developmental-stage dependent effects of glucocorticoids in regulating steroidogenesis in chicken

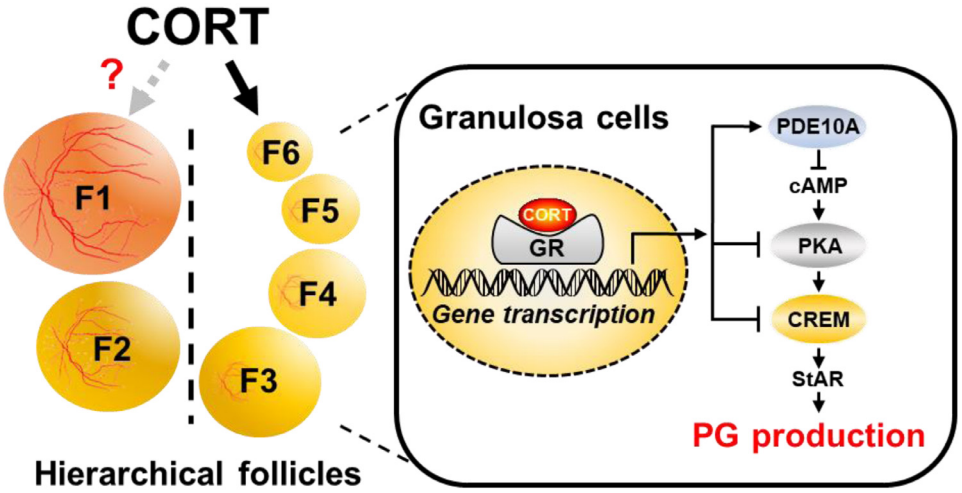


**Figure 8.** Proposed model for CORT action on chicken ovarian GCs from hierarchical follicles at different developing stages. CORT inhibits PG production in GCs from small F5-6, F4, and F3 follicles, but not in GCs from big sizes F2 and F1 follicles. The intracellular GR-binding CORT primarily inhibits PG production via blocking StAR expression in cAMP-dependent manner, i.e., promoting *PDB10A* expression but suppress the mRNA accumulation of both *PRKAR2A* and *CREM*.

hierarchical GCs, actualized through a cAMP-StAR dependent manner. Undoubtedly, the evidence presented here will pave the way to understanding the etiology of stress-triggered ovarian dysfunctions and laying pause in chicken.

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## DISCLOSURES

The authors declare no conflicts of interest.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102379.

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