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Growth differentiation factor 9 promotes follicle-stimulating hormone-induced progesterone production in chicken follicular granulosa cells

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ABSTRACT

The function of oocyte-derived growth differentiation factor 9 (GDF9) in ovarian follicles has thus far been poorly defined in avian species compared with the defined function in mammals. Our aim here is to investigate the effects of GDF9 on steroidogenesis and on chicken ovarian granulosa cell (GC) mitosis. Primary GCs from both prehierarchical (6–8 mm in diameter, phGCs) and preovulatory follicles (F1–F5, poGCs) were cultured in the presence or absence of the GDF9 protein. The progesterone (P4) levels in the culture medium were then measured by radioimmunoassay (RIA), and the expression levels of steroidogenesis genes were detected by quantitative PCR. We found that GDF9 alone showed no significant effect on the P4 levels by regulating the expression of steroidogenesis genes, such as *STAR*, *CYP11A1* and *HSD3B*. Further experiments indicated that GDF9 promoted follicle-stimulating hormone (FSH)-induced P4 production and *STAR* expression. GDF9 also rescued the FSH-induced decrease of FSH receptor (*FSHR*) expression but had no effect on the forskolin-induced P4, *STAR* and forskolin-inhibited *FSHR* expression levels, suggesting that GDF9 might achieve its regulatory role of P4 by enhancing *FSHR* and *STAR* expression. In addition, GDF9 also promoted GC cell cycle progression, regulated the gene transcription of related genes, potentiated DNA replication and inhibited apoptosis. Interestingly, these effects differed between the phGCs and the poGCs. To our knowledge, this is the first report that illustrates the function of GDF9 on chicken GCs and the effects on ovarian steroidogenesis. Our findings highlight the regulation of central oocytes on the surrounding granulosa cells and emphasize the interaction between paracrine signals and endocrine hormones on ovarian progesterone production; these findings contribute to the understanding of the development of avian ovarian follicles.

1. Introduction

The growth, development and function of chicken ovarian follicles determine the laying performance of chickens. When chickens are preparing to lay eggs, the chicken ovaries develop in an orderly and progressive manner for all stages of follicles, which can be broadly divided into prehierarchical follicles and preovulatory follicles (Johnson, 2015). Follicle selection occurs approximately once a day; during this process, the follicles are recruited into a preovulatory hierarchy from a cohort of small yellow follicles (SYF) of 6–8 mm in diameter (Y. Wang et al., 2017). The selected follicle will continue to quickly grow and develop from F5/6 (the newly selected follicle) to F1 (the largest yellow follicle) to over 30 mm in diameter within several days and will then be ovulated.

After follicle selection, the granulosa cells (GCs) that surround the oocyte continue to rapidly proliferate to meet the demand of the enlarging follicle and dramatically upregulate progesterone (P4) production; these events mark the two main differences between the GCs from prehierarchical follicles (phGCs) and those from preovulatory follicles (poGC). As is known in mammals, P4 participates in various physiological processes, including follicular function and placental development (Taraborrelli, 2015). Due to the different reproductive physiologies of avian and mammalian species, chickens predominantly produce P4 by poGCs, where in mammals, P4 is synthesized mainly by the corpus luteum and the placenta (Lee et al., 1998). Though differences exit, birds and mammals share the same steroidogenesis pathway, which converts the primary substrate cholesterol into progesterone and utilizes the following proteins: StAR (steroidogenic acute regulatory

Abbreviations: GDF9, growth differentiation factor 9; FSH, follicle-stimulating hormone; FSHR, follicle-stimulating hormone receptor; P4, progesterone; STAR, steroidogenic acute regulatory protein; CYP11A1, P450 side chain cleavage enzyme; HSD3B, 3β-hydroxysteroid dehydrogenase; GC, granulosa cell; phGC, granulosa cell from prehierarchical follicles; poGC, granulosa cell from preovulatory follicles; RIA, radioimmunoassay; EdU, 5-ethynyl-2′-deoxyuridine

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protein), P450scc (P450 side chain cleavage enzyme) and 3β-HSD (3β-hydroxysteroid dehydrogenase) (Gómez et al., 1998; Kato et al., 1995; Miller and Auchus, 2011). A variety of endocrine hormones from the hypothalamic-pituitary axis, such as follicle-stimulating hormone (FSH), and the paracrine and autocrine signals from the ovarian follicles are involved in the regulation of P4 production. FSH, secreted by the pituitary gland, plays an essential role in female ovarian development and function. As is reported in both mammals and birds, the binding of FSH to FSH receptors (FSHR) induces intracellular cAMP production and the activation of the PKA signaling pathway, and thus, results in the regulation of receptor cell functions (Hunzicker-Dunn and Maizels, 2006; Ocon-Grove et al., 2012; Woods and Johnson, 2005).

Growth differentiation factor 9 (GDF9), one of the oocyte-derived factors, plays an important role in ovary development and maturation, which has been well studied in mammals (Otsuka et al., 2011; Paulini and Melo, 2010). Mice deficient in GDF9 failed to develop ovarian stages beyond the primary, one-layer follicle, and thus, the females were sterile (Dong et al., 1996). Recent findings of the associations between several SNPs in *GDF9* and egg production traits in two Chinese local chicken breeds emphasized the necessary role of GDF9 in the development of hen ovaries (Huang et al., 2015; Qin et al., 2015). Previous studies on chicken ovaries have shown that the expression of GDF9 is primarily during follicular development and have suggested a stimulatory effect on prehierarchical GC proliferation (Elis et al., 2007; Johnson et al., 2005), although the effect on hierarchical/preovulatory follicles remains unclear. It has been reported in mammals that GDF9 can regulate the levels of basal and/or gonadotropin-induced progesterone secretion by granulosa cells, although the effect differs in various animals (Hickey et al., 2005; Spicer et al., 2006; Vitt et al., 2000). However, little is known about whether GDF9 exerts an effect on ovarian steroidogenesis in avian species.

To explore the function of GDF9 on GC progesterone synthesis from preovulatory follicles and to explore whether the mitotic actions of phGCs and poGCs differ in response to GDF9 stimulation, we conducted the current study. It was found that oocyte-derived GDF9 alone had no significant effect on the levels of basal P4 production by poGCs but did promote FSH-induced P4 production and regulated the gene expression of steroidogenesis-related genes. Our findings emphasize the paracrine effect of oocytes on the surrounding granulosa cells and the interaction between factors from the ovary and the pituitary gland. In addition, we also assessed the differential effects of GDF9 on the cell cycle, DNA replication and apoptosis between phGCs and poGCs. Our results provide a better understanding of GDF9 action on the function of chicken granulosa cells, and thus, contribute to the knowledge of avian ovarian follicular biology.

2. Materials and methods

2.1. Animals and reagents

Sexually matured hens (30–40 wk. of age) with continuous egg laying were purchased from the Xinhua Chicken Farm (Hubei, China) and were maintained in cages with free access to food and water. The hens were sacrificed by cervical dislocation, and the prehierarchical small yellow follicles (SYF, 6–8 mm in diameter) and the preovulatory follicles (F1–F5/6, 10–35 mm in diameter) were removed from the ovaries. For each experiment, 3–5 hens were sacrificed for sample collection. The sample collection was approved by the Ethics Committee of Huazhong Agricultural University, and the experiments were carried out in accordance with the approved guidelines. Recombinant human (rh) GDF9 and rhFSH were purchased from R&D systems Inc. (USA) and forskolin was purchased from Tocris (USA). The biologically active C-terminal region of the human GDF9 protein (accession: O60383.1, 316–454 aa) had an 82% homology with the chicken GDF9 (NP_996871.2, 321–454 aa).

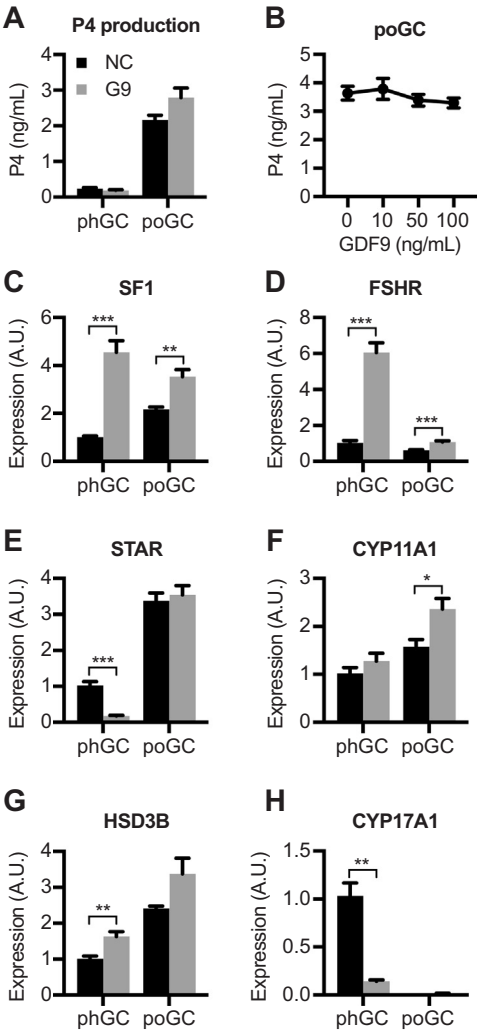

Fig. 1. The effect of GDF9 on progesterone (P4) production and steroidogenesis gene expression in chicken granulosa cells. (A) Primary granulosa cells from prehierarchical (phGCs) and pro-ovulatory (poGCs) follicles were cultured for 24 h in the presence or absence of GDF9 (50 ng/mL, indicated as G9 or NC), and the progesterone secretion in the medium was detected by RIA (n = 6). (B) The effect of the dose of GDF9 (0–100 ng/mL) on poGC P4 production (n = 4) is shown. The mRNA expression levels of *SF1* (C), *FSHR* (D), *STAR* (E), *CYP11A1* (F), *HSD3B* (G) and *CYP17A1* (H) were measured by qPCR. The data are presented as the mean ± SEM (n = 6). Two-tailed Student's t-tests were used to analyze the significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

2.2. Granulosa cell culture and treatment

Granulosa cells (GCs) from prehierarchical follicles (phGCs, from SYF with 6–8 mm in size) and preovulatory follicles (poGCs, from mixed F1–F5/6 follicles) were collected according to previously reported methods (Gilbert et al., 1977; J. Wang et al., 2017). The cell viability was determined by trypan blue staining, and the cell density was calculated by an automated cell counter (Countstar IC1000, Shanghai Ruiyu Biotech Co., LTD). For EdU assays, the GCs were seeded into 96-well plates at a density of 50,000 viable cells/well (n = 5). In the cell cycle and apoptosis assays, the GCs were seeded into 6-well plates at a density of 500,000 viable cells/well (n = 3). For P4 detection and RNA extraction, the GCs were seeded into 24-well plates at a density of 250,000 viable cells/well. The primary GCs were precultured for 12–16 h with Medium 199 (Gibco, USA) and 5% FBS (AusGeneX, Australia) until they were treated for 24 h with M199 and 1xITS (Sigma, USA) in the presence or absence of rhGDF9, rhFSH and/or forskolin depending on the experimental group.

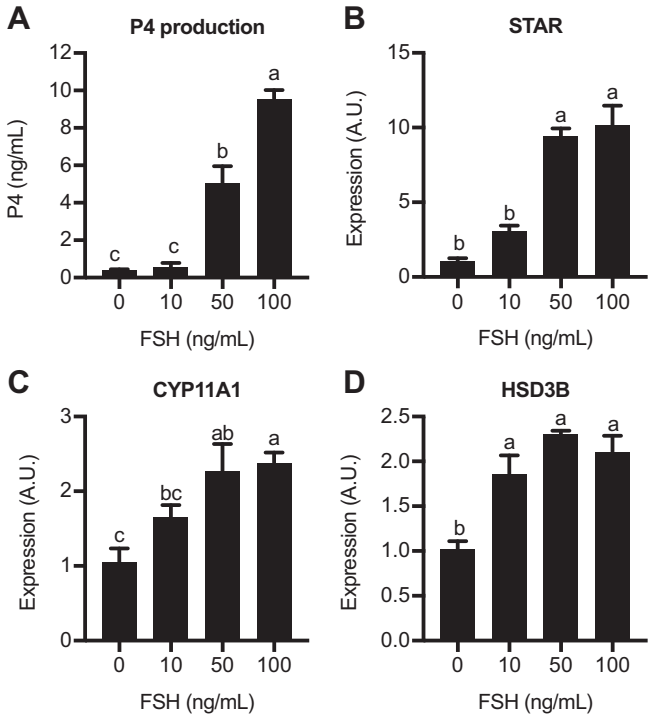

Fig. 2. FSH induces P4 production and steroidogenesis gene expression by poGCs in a dose-dependent manner. (A) FSH induces poGC progesterone production in a dose-dependent manner. Primary granulosa cells from preovulatory follicles (poGCs) were cultured for 24 h with various concentrations of FSH. P4 was measured by RIA, and the mRNA levels of *STAR* (B), *CYP11A1* (C) and *HSD3B* (D) were detected by qPCR. The data are presented as the mean ± SEM (n = 4). Multiple comparisons were conducted by Duncan's post hoc comparison, and the different subscripts indicate that the difference is significant ($P < 0.05$).

To detect the effects of GDF9 on P4 production (Fig. 1A), phGCs and poGCs were seeded into 24-well plates at a density of 250,000 viable cells/well and were then treated for 24 h with or without GDF9 (50 ng/mL). In an additional GDF9 dosage assay (Fig. 1B), poGCs were seeded into a 24-well plate at the former density and were treated for 24 h with a variety of GDF9 concentrations (0, 10, 50, 100 ng/mL). In the FSH dosage experiment (Fig. 2), poGCs were cultured in 24-well plates at a density of 250,000 viable cells/well with various FSH concentrations (0, 10, 50, 100 ng/mL; or 0, 0.175, 0.875, 1.750 IU/mL). Then, moderate 50 ng/mL FSH was selected for the following assay with a GDF9 gradient (0, 10, 50, 100 ng/mL) (Fig. 3). For the forskolin-stimulating assay (Fig. 4A), poGCs were cultured in 24-well plates at a density of 250,000 viable cells/well with forskolin gradients (0, 10, 50, 100 μM). PoGCs were cultured with a combined treatment of forskolin (50 μM) and GDF9 (0, 10, 50, 100 ng/mL). The P4 and gene expression levels were measured by RIA as described in Section 4.3 and by qPCR as described in Section 4.4, respectively.

2.3. Progesterone radioimmunoassay

The culture medium was collected from the individual wells after gentle centrifugation and was stored at −20 °C for 2–7 days before being transported in dry ice to Beijing North Institute of Biological Technology for concentration determinations. The concentration of progesterone in the culture media supernatant was measured by radioimmunoassay (RIA) with the detection range of 0.2–100 ng/mL; the intra and interassay coefficients of variation were 10% and 15%, respectively.

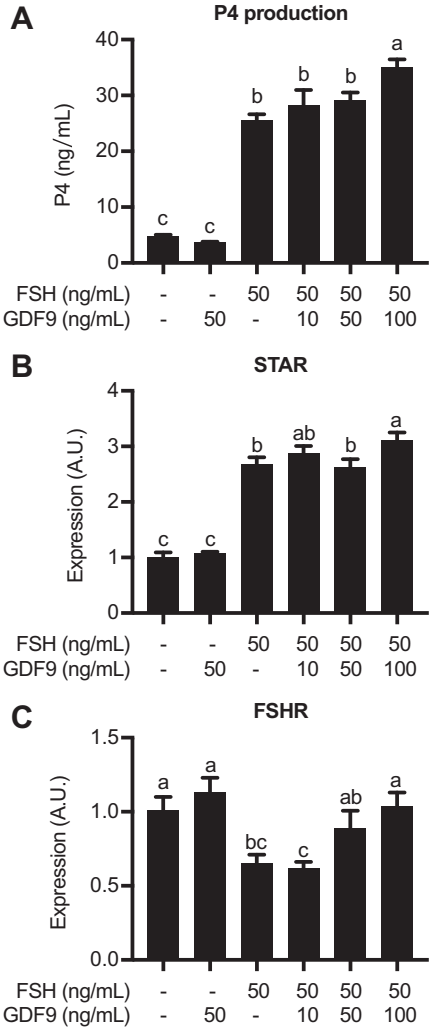

Fig. 3. GDF9 promotes FSH-induced P4 production and the mRNA expression of *STAR* and *FSHR* in poGCs. (A) GDF9 promotes FSH-induced P4 production. Primary granulosa cells from preovulatory follicles (poGCs) were cultured for 24 h with various treatments. P4 was measured by RIA, and the mRNA levels of *STAR* (B) and *FSHR* (C) were detected by qPCR. The data are presented as the mean ± SEM (n = 4). Multiple comparisons were conducted by Duncan's post hoc comparison, and the different subscripts indicate that the difference is significant ($P < 0.05$).

2.4. Quantitative real-time PCR

The total RNA was extracted from the cultured primary granulosa cells using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA was reverse-transcribed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) following the manufacturer's recommended procedure. The RNA concentration was first quantified by spectrophotometry (NanoDrop 2000, Thermo Scientific, USA) and was then adjusted to 1 μg per reaction. The RT primer mix was a mix of oligo-dT primers and random 6-mers. The resulting cDNA was stored at −20 °C. Real-time PCR was conducted on a CFX-384 (Bio-Rad, USA) with a 10 μL volume that contained 5 μL of 2xTaq™ Universal SYBR Green Supermix (Bio-Rad), 0.2 μL each of the forward and reverse primers (10 μM, see Table 1), and 4.6 μL of cDNA (approximately 100 ng for each reaction). The melting curves and product sequences were investigated to confirm the specificity of each primer. The relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) method, and the mean levels of *ACTB* and *GAPDH* were used as an internal control (Jing et al., 2018; J.

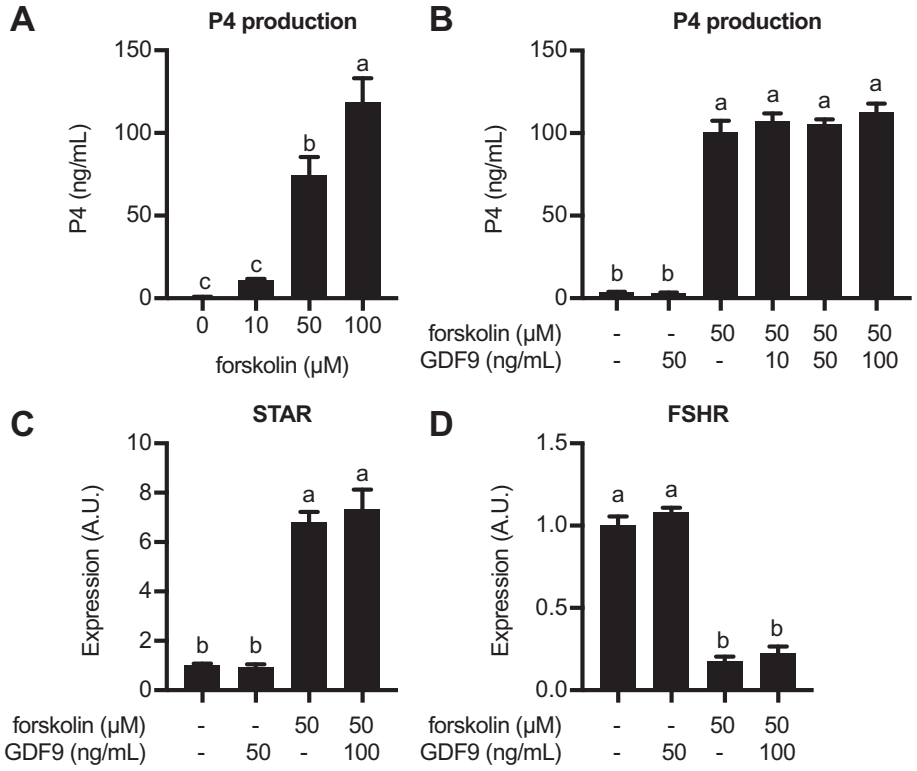

Fig. 4. The effects of GDF9 and forskolin on P4 production by poGCs. (A) Forskolin induces poGC progesterone production in a dose-dependent manner. (B) GDF9 shows no effect on forskolin-induced P4 production. The granulosa cells from preovulatory follicles (poGCs) were cultured 24 h before P4 detection and the qPCR measurements of *STAR* (C) and *FSHR* (D) expression. The data are presented as the mean ± SEM (n = 4). Multiple comparisons were conducted by Duncan's post hoc comparison, and the different subscripts indicate that the difference is significant ($P < 0.05$).

Wang et al., 2017). The samples that contained six biological replicates were amplified in triplicate.

2.5. Flow cytometry analysis for detecting the cell cycle and apoptosis levels

After 16 h of preculture in 6-well plates at a density of 500,000 viable cells/well, the primary granulosa cells were cultured 24 h in the presence or absence of GDF9 (50 ng/mL). The collected cells were then stained with propidium iodide (PI) solution (50 μg/mL PI and 100 μg/mL RNase A in PBS) and were then subjected to cell cycle analysis with Cytomics FC 500 (Beckman, USA). The data were collected and analyzed with the ModFit LT software. The extent of apoptosis was measured using Annexin V/ PI double staining. Briefly, 100 μL of binding buffer containing 2.5 μL of Annexin V-FITC and 1 μL of PI was added to the cell suspension, which was then incubated for 30 min in the dark. Then, the samples were analyzed with Cytomics FC 500, and the apoptosis rate was calculated as a percentage.

2.6. EdU assay

The primary phGCs and poGCs were precultured for 16 h in 96-well plates at a density of 50,000 viable cells/well before 5-ethynyl-2′-deoxyuridine (EdU) addition. EdU (10 μM, Cell Light EdU DNA Imaging Kit, Guangzhou RiboBio, China) was added in the presence or absence of GDF9 (50 ng/mL), and the cells were then cultured for an additional 24 h. The cells were then stained according to the manufacturer's protocol. Briefly, the cells were fixed in 4% paraformaldehyde at room temperature for 30 min, were washed in a shaker with glycine (2 mg/mL) for 5 min, then were treated with 0.2% Triton X-100 for 10 min. After permeabilization, the cells were reacted with 1 × Apollo reaction buffer. The cell nuclei were counterstained with Hoechst 33,342 and were visualized under a fluorescence microscope (Eclipse, Nikon, Japan). The cells with different staining were counted by Image-Pro Plus 6.0 (Media Cybernetics, USA) with 5 observers per group, and the EdU-positive cell percentage was finally calculated as (EdU add-in

Table 1
Primers for qPCR.

Gene Symbol	Accession Number	Forward Sequence (5′-3′)	Reverse Sequence (5′-3′)	Product (bp)
GAPDH	NM_204305	GAGGGTAGTGAAGGCTGCTG	CACAACACGGTTGCTGTATC	199
ACTB	NM_205518	TGGGTATGGAGTCCTGTGGT	AGGGCTGTGATCTCCTTCTG	160
CDK2	NM_001199857	ACGTGATCCACACGGAGAAC	GCAGCTGGAACAGGTAGCTC	132
CCNE1	NM_001031358	AGGTTTATGGCAACACAACAGAA	AACTGGTGCAACTTTGGTGG	116
CDK6	NM_001007892	CCAGACCCGCACAACCTATT	TCTTGGCTGGATTGAACGCT	96
CCND1	NM_205381	ATAGTCGCCACTTGGATGCT	AACCGGCTTTTCTTGAGGGG	122
CDKN1A	NM_204396	TACGAAGCAATGCCGAGTCT	TCAGTCCCTTCCGTGGTCTT	116
CDKN1B	NM_204256	GAGCCCGAGACGACATCAAA	TCCCATGGAGACCGACGATA	133
TP53	NM_205264	GTCCCATCCACGGAGGATTA	CCAGGCGGCAATAGACCTTA	124
ATM	NM_001162400	TCCCGCATTTTCTCGCAGAT	TCAAGGGAAGAGGCGTTGAC	123
SF1	NM_205077	CGCTGAGCCGCATCGAG	TTGTTGTTCTGCACGGTCCT	165
STAR	NM_204686	GTCCCTCGCAGACCAAGT	TCCCTACTGTTAGCCCTGA	196
CYP11A1	NM_001001756	GTGGACACGACTTCCATGACT	GAGAGTCTCCTTGATGGCGG	174
CYP17A1	NM_001001901	TGCTCCCTCTGCTTCAACTC	ATCCATCAGGTCCCTCACAG	255
HSD3B	NM_205118	TGGAAGAAGATGAGGCGCTG	GGAAGCTGTGTGGATGACGA	185
FSHR	NM_205079	GAGCGAGGTCTACATACA	GCACAAGCCATAGTCA	281

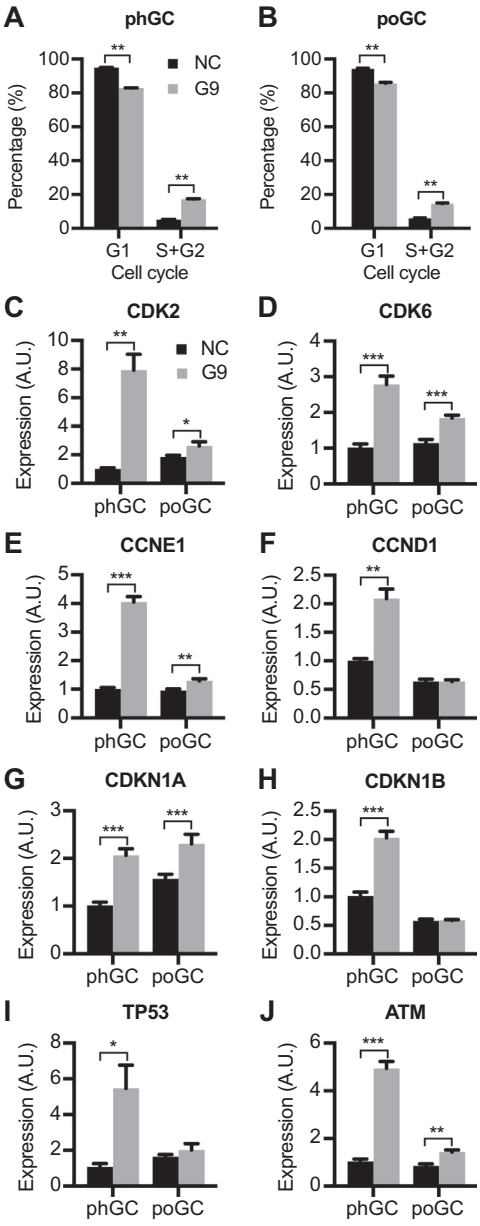

Fig. 5. GDF9 promotes the G1 to S/G2 transition of chicken granulosa cells *in vitro*. Primary granulosa cells from prehierarchical (phGCs) and preovulatory (poGCs) follicles were cultured for 24 h in the presence or absence of GDF9 (50 ng/mL, indicated as G9 or NC). A summary of the cell phase distribution is shown in A (phGCs) and B (poGCs). The data are presented as the mean ± SEM (n = 3). GDF9 regulates the mRNA expression of the cell cycle-related genes *CDK2* (C), *CDK6* (D), *CCNE1* (E), *CCND1* (F), *CDKN1A* (G), *CDKN1B* (H), *TP53* (I) and *ATM* (J). The means of *ACTB* and *GAPDH* were used as an internal control. The expression data are presented as the mean ± SEM (n = 6). Two-tailed Student's t-tests were used to analyze the significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

cells/Hoechst stained cells) × 100%.

2.7. Statistical analysis

All data were processed according to the individual procedures described above. A paired comparison was performed by two-tailed Student's *t*-tests using Microsoft Excel 2016, and the significance is indicated in the figures ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns not significant). Multiple comparisons were conducted by Duncan's post hoc comparison using SPSS statistics (version 23.0 for mac, IBM, USA). $P < 0.05$ was considered statistically significant. All numeric results were visualized using GraphPad Prism 7 (GraphPad software, USA).

3. Results

3.1. GDF9 alone exerts no significant effect on progesterone production but regulates the expression of steroidogenesis genes

A progesterone RIA assay showed that compared with progesterone secretion by poGCs, the progesterone secretion by phGCs was nearly undetectable (Fig. 1A). GDF9 failed to induce progesterone production in phGCs but tended to promote P4 production minimally in poGCs ($P = 0.05$, Fig. 1A). Further assays confirmed that GDF9 exerted no significant effect on the basal P4 levels in cultured poGCs, with concentrations varying from 10 ng/mL to 100 ng/mL (Fig. 1B). Meanwhile, the qPCR results revealed that in poGCs, GDF9 upregulated the mRNA expression of *SF1*, *FSHR*, *CYP11A1* and *HSD3B*, with no effect on the levels of *STAR* and *CYP17A1* (Fig. 1C–H). In phGCs, GDF9 elevated the mRNA levels of *SF1*, *FSHR*, *CYP11A1* and *HSD3B* but inhibited *STAR* and *CYP17A1* expression.

3.2. FSH induces P4 production and steroidogenesis gene expression in a dose-dependent manner by cultured poGCs

Fig. 2A shows that FSH stimulated the progesterone production by cultured poGCs at a range of 10–100 ng/mL in a dose-dependent manner. Consistently, the mRNA expression levels of the steroidogenesis genes *STAR* (B), *CYP11A1* (C) and *HSD3B* (D) were elevated by increasing the dose of FSH. In response to a 50 ng/mL FSH stimulation, the P4 level and the *STAR* mRNA were both upregulated ~10-fold, while the levels of *CYP11A1* and *HSD3B* were elevated more than 2-fold compared to the levels of the respective controls (0 ng/mL FSH). There were no significant differences in the mRNA expression levels of *STAR*, *CYP11A1* and *HSD3B* between the FSH treatments of 50 ng/mL and 100 ng/mL. Consequently, 50 ng/mL of FSH was chosen for the following experiments.

3.3. GDF9 promotes FSH-induced but not forskolin-induced P4 production

Consistent with the previous results, FSH dramatically induced P4 production, while GDF9 had no significant effect on P4 production (Fig. 3A). Moreover, compared to the production of the group treated with FSH alone, the granulosa cells produced more P4 with the combined treatment of FSH (50 ng/mL) and GDF9 (100 ng/mL), while lower concentrations of GDF9 (10–50 ng/mL) showed no significant effect on this FSH-induced P4 accumulation. The qPCR profiles showed that a high dose of GDF9 also tended to increase *STAR* expression when cotreated with FSH compared with the expression when treated with FSH alone (Fig. 3B). Meanwhile, GDF9 dose-dependently rescued *FSHR* expression, which was downregulated by FSH treatment alone (Fig. 3C).

As a potent activator of the enzyme adenylate cyclase (AC), which increases the intracellular cAMP concentration, a twenty-four h culture with forskolin induced the production of P4 by poGCs in a dose-dependent manner (Fig. 4A); thus, 50 μM of forskolin was chosen as the effective concentration to stimulate P4 in further experiments. Additional assays showed that various concentrations of GDF9 (10–100 ng/mL) had no effect on forskolin-induced P4 production (Fig. 4B). Co-treatment assays showed that GDF9 had no significant effect on either the expression levels of forskolin-induced *STAR* or forskolin-reduced *FSHR* (Fig. 4C and D).

3.4. GDF9 promotes the cell cycle progression of granulosa cells cultured in vitro

Flow cytometry showed that GDF9 promoted the G1 to S/G2

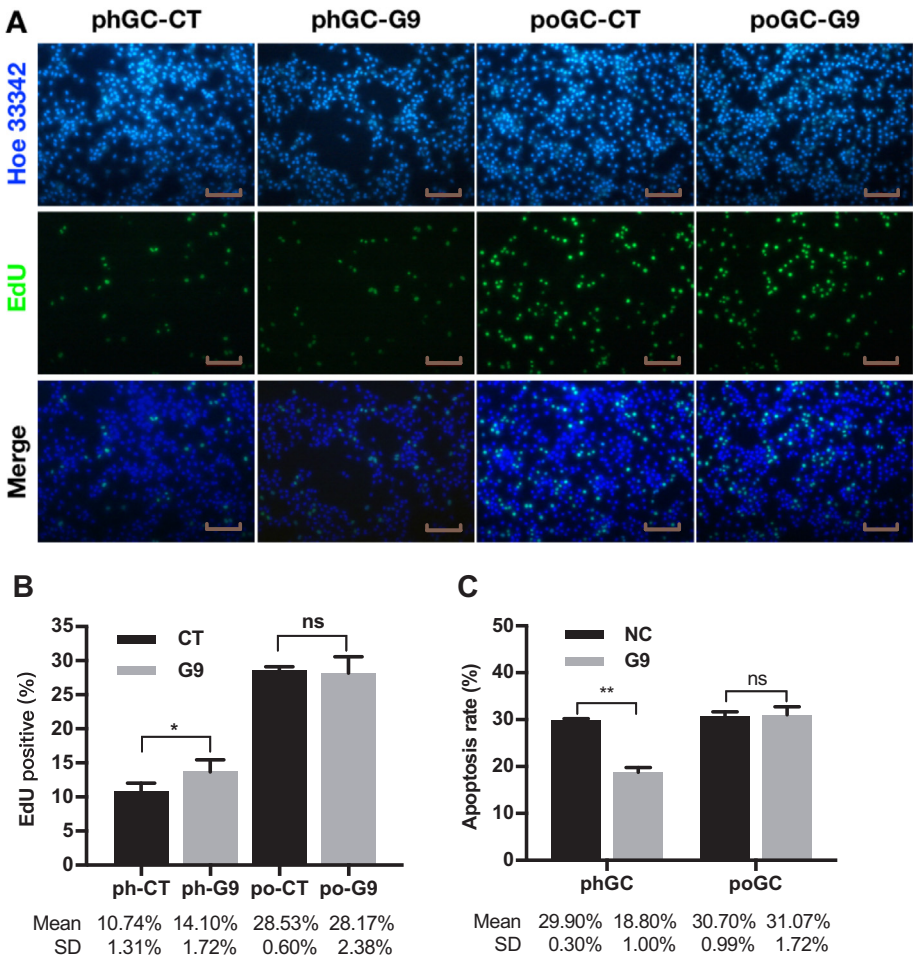

Fig. 6. The effects of GDF9 on the DNA replication and apoptosis of phGCs and poGCs *in vitro*. (A) Representative images of Hoechst 33342-stained cells (top, indicating cell nuclei), EdU-stained cells (middle, indicating DNA replication) and the merged images (bottom) of phGCs and poGCs. Primary granulosa cells from prehierarchical (phGCs) and preovulatory (poGCs) follicles were cultured for 24 h with EdU-containing medium in the presence or absence of GDF9 (50 ng/mL, indicated as G9 or CT). Scale bar = 100 μm. (B) A summary of the EdU add-in cell percentage of granulosa cells. The data are presented as the mean ± SD (n = 5). (C) The GDF9 effect on the apoptosis of phGCs and poGCs. Apoptosis was analyzed by flow cytometry, and the number of apoptotic cells (including Annexin V+/PI− and Annexin V+/PI+) is presented as the mean ± SD (n = 3). Two-tailed Student's t-tests were used to analyze significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$, ns not significant).

transition during cell cycle progression in both phGCs and poGCs (Fig. 5A-B). Culturing phGCs with GDF9 decreased the proportion of the G1 phase by ~12% and increased the proportions of the S and G2 phases by ~7% and ~5%, respectively. GDF9 also increased the poGC portion in the S/G2 phase by 8.63%. Furthermore, quantitative PCR showed that the mRNA levels of *CDK2, CCNE1, CDK6* and *CCND1* in both the phGCs and the poGCs were significantly elevated by GDF9 treatment (Fig. 5C–F), as were the gene expression levels of *CDKN1A, CDKN1B, TP53* and *ATM* (Fig. 5G–J). It was noted that the promoting effect of GDF9 on phGC mRNA expression was obviously larger than that on poGC mRNA expression, at least for the genes examined here.

3.5. GDF9 enhances DNA synthesis and inhibits the apoptosis of phGCs but not of poGCs in vitro

A comparison between the groups in the presence and absence of GDF9 indicated that in the GDF9-treated group, the proportion of cells that incorporated EdU was significantly elevated in phGCs ($P < 0.05$) but not in poGCs (Fig. 6A and B). An EdU assay also showed the control poGCs (po-CT: 28.53% ± 0.60%) had more DNA synthesis activity than that of the control phGCs (ph-CT: 10.74% ± 1.31%).

Moreover, we examined the apoptosis of GCs using an Annexin V-PI apoptosis detection kit and found that the percentage of apoptosis was 18.75% ± 1.06% in phGCs exposed to GDF9 compared with 29.90% ± 0.28% in the NC control (Fig. 6C). However, GDF9 showed no significant effect on poGC apoptosis, with 30.70% ± 10.99% and 31.07% ± 1.72% in the NC and G9 groups, respectively.

4. Discussion

Since Johnson first reported that anti-GDF9 antiserum blocked the proliferating effect of oocyte-conditioned medium (OCM) on hen granulosa cells (Johnson et al., 2005), little knowledge has been gained on the function of GDF9 in the chicken ovaries, especially about sex hormone synthesis. Herein, for the first time, we explored the role of GDF9 in chicken ovarian progesterone (P4) production and the regulation of steroidogenesis genes. We found that GDF9 alone showed no significant effect on the basal P4 production by poGCs cultured *in vitro* but did promote FSH-induced P4 secretion. Interestingly, FSH stimulation decreases *FSHR* expression, and GDF9 can rescue this down-regulated *FSHR* to a normal level. It is hypothesized that *FSHR* upregulation by GDF9 might be involved in GDF9-enhanced FSH-induced P4 production, which requires further validation.

Progesterone is one of the key sex hormones that regulates reproductive physiology and female behavior in mammals. It exerts various effects on multiple organs and tissues, including the ovaries, endometrium, oviduct, breasts and pituitary gland (Taraborrelli, 2015). In contrast to mammals, chickens lack the processes of corpus luteum formation and embryo implantation, and chickens have a unique reproductive physiology in which P4 functions differentially than in other species. The plasma P4 level elevates 6–8 h before chicken ovulation and peaks approximately 2–3 h after ovulation, indicating its role in regulating avian ovulation (Etches, 1979). P4 also regulates the hen oviduct secretion of proteins into the egg whites, and this affects embryonic development during the following incubation (Joensuu et al., 1991). A recent study discovered that P4 could trigger chicken germ cell meiotic initiation (Mi et al., 2014).

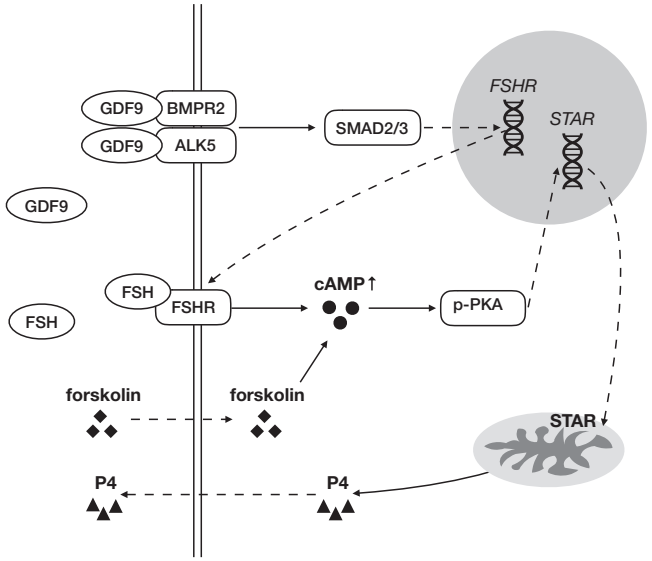

Fig. 7. Schematic representation of the hypothesized mechanism of GDF9-promoted FSH-induced progesterone secretion. FSH binding to the membrane receptor FSHR increases the intracellular cAMP levels and potentiates PKA (protein kinase A) phosphorylation to modulate the gene expression and to induce P4 production. Forskolin, a potent PKA activator, also induces *STAR* expression and P4 production. GDF9, which is proposed to form dimers and then bind to type I and type II receptors ALK5 and BMPR2 to activate SMAD2/3 signaling, upregulates *FSHR* expression and enhances the P4 secretion of granulosa cells in response to FSH stimulation.

In the mammalian urogenital system, P4 is synthesized predominantly by the corpus luteum during the normal ovarian cycle and by the placenta during pregnancy. While chicken GCs from preovulatory follicles are the main source of P4, it is believed that the basic enzymatic pathways that catalyze cholesterol into progesterone remain the same as those in mammals, including StAR, P450scc and 3β-HSD. Our RIA results show that after follicle selection, poGCs start a larger amount of P4 synthesis compared to that in phGCs (NC groups in Fig. 1A), with the consistent upregulation of *SF1, STAR, CYP11A1* and *HSD3B* and the downregulation of *CYP17A1* (Fig. 1). SF1 protein (namely, NR5A1) acts as a master regulator and a transcription factor that controls the expression of many downstream steroidogenesis genes, and thus, may affect the production of the final sex hormones (Yazawa et al., 2015). The *CYP17A1* gene, which encodes the P450c17 enzyme, is responsible for the conversion of progestin to androgen. Our results show that GDF9 alone is not capable of regulating the basal progesterone production by poGCs (Fig. 1A and B). This finding is inconsistent with those from previous studies in mice and rats, which suggested that rodent GDF9 alone had a role in stimulating progesterone production by granulosa cells (Elvin et al., 2000; Vitt et al., 2000); these findings may indicate the differences between birds and mammals. However, in poGCs, GDF9 is sufficient to significantly upregulate *SF1*, *CYP11A1* and *HSD3B* expression, with no effect on *STAR* and *CYP17A1* expression.

As a gonadotrophin from the pituitary gland, follicle-stimulating hormone (FSH) has a dose-dependent inducing effect on progesterone secretion (Fig. 2A); this accompanies the dose-related upregulation of *STAR*, *CYP11A1* and *HSD3B* and is consistent with results from previous studies (Johnson and Lee, 2016). Among these steroidogenesis genes, the *STAR* mRNA level was elevated ~10-fold by poGCs in response to the higher concentration of FSH (Fig. 2B), while *CYP11A1* and *HSD3B* exhibited increased expression levels by over 2-fold (Fig. 2C and D). It seems that the augmentation of *STAR* expression is related to the FSH-induced P4 production, which suggests that *STAR* is a promising marker of P4 production. There were no significant differences in the mRNA expression levels of *STAR*, *CYP11A1* and *HSD3B* between the FSH treatments of 50 ng/mL and 100 ng/mL; this indicates that enzymatic activity enhancements, rather than the corresponding gene transcription changes, may be involved.

Previous experiments showed that recombinant rat GDF9 inhibited FSH-induced P4 production by both rat and cattle GCs *in vitro* (Spicer et al., 2006; Vitt et al., 2000) and that recombinant mouse GDF9 inhibited porcine mural GC progesterone production (Hickey et al., 2005). Here, we observed an opposite promoting effect of GDF9 on FSH-induced chicken P4 production (Fig. 3A), illustrating the differences in GDF9 function on endocrinal gonadotrophins between birds and mammals. The differences in the experimental conclusions here may result from not only the origins of GDF9 proteins (human or rodent) but also the sources of the target granulosa cells (chicken or mammalian); the same brand of rhGDF9 used in the present study was used to previously inhibit FSH-stimulated P4 production by bovine granulosa cells (Spicer et al., 2006). The signaling pathways that involve GDF9 function might be different and complicated in various animals, and the underlying molecular basis requires further investigation.

As a potent activator of adenylyl cyclase, forskolin is commonly applied in *in vitro* experiments to increase the intracellular cAMP concentration (Sechman et al., 2011). In addition, its role in stimulating steroidogenesis has been investigated in several animal models (Asem and Hertelendy, 1983; Otsuka, 2001; Solovyeva et al., 2000). Our experiment also shows that forskolin is sufficient to induce large amounts of P4 production by hen poGCs in a dose-dependent manner (Fig. 4A), indicating the participation of protein kinase A (PKA) in P4 synthesis. In striking contrast, GDF9 did not significantly affect forskolin-induced P4 accumulation (Fig. 4B), which probably suggests that the promoting effect of GDF9 on FSH action is upstream of cAMP signaling. Further investigations showed that GDF9 rescued FSH-decreased *FSHR* mRNA levels in a dose-dependent manner (Fig. 3C) but did not rescue the forskolin-inhibited *FSHR* expression (Fig. 4D). Based on these results, we hypothesize that GDF9 promotes FSH-induced P4 production through its ability to elevate *FSHR* expression and affect the downstream pathway (Fig. 7); these hypotheses require further investigation.

In the second part of our study, we surveyed the effect of GDF9 on GC mitosis. An EdU assay and flow cytometry analyses of the cell cycle and apoptosis showed that GDF9 exerts an anti-apoptotic effect on phGCs by promoting DNA synthesis and cell cycle progression. All the results are logically consistent with the previous study, which claimed that there was a stimulatory effect by chicken GDF9 on GC proliferation (Johnson et al., 2005). To explore the molecular mechanism, quantitative PCR was conducted; qPCR indicated that the mRNA levels of *CDK2*, *CCNE1* (cyclin E1), *CDK6* and *CCND1* (cyclin D1) were upregulated by GDF9 stimulation (Fig. 5). CDK2 forms a complex with cyclin E and controls the G1-S phase transition of the cell cycle, while CDK6 controls the G1 phase of the cell cycle in complex with cyclin D (Ruijtenberg and van den Heuvel, 2016). The elevated expression of these genes indicates that GDF9 may regulate the cell cycle by enhancing both the actions of the CDK2/cyclin E and CDK6/cyclin D complexes. Interestingly, the mRNA levels of CDK/cyclin complex, *CDKN1A* and *CDKN1B* inhibitors and the levels of their upstream regulators (*TP53* and *ATM*) were also upregulated by GDF9 treatment, which may indicate that feedback regulations maintain the homeostasis of cell cycle progression. An alternative reasonable explanation exists because ATM, TP53 and CDKN1A also participate in apoptosis, DNA damage repair and other cellular processes (Aubrey et al., 2016; Serrano et al., 2012).

In addition to progesterone regulation, differences in the effects of GDF9 on cell functions between phGCs and poGCs are also observed here. According to previous studies (Elis et al., 2007; Johnson et al., 2005), the expression of oocyte-derived GDF9 is more enriched in prehierarchical follicles than in preovulatory follicles. Our *in vitro* assays revealed a more sensitive response of phGCs to GDF9 stimulation than the response of poGCs in terms of cell cycle, DNA replication and apoptosis. QPCR monitoring of cell cycle-related genes also illustrated

larger expression changes in phGCs than those in poGCs, which is consistent with cell cycle progression (Fig. 5). These findings suggest that GDF9 may be of greater importance in mitotic and apoptotic actions in prehierarchical follicles than their importance in preovulatory follicles.

In conclusion, GDF9 promotes FSH-induced P4 production in chicken granulosa cells and regulates *FSHR* and *STAR* gene expression. Furthermore, GDF9 also participates in GC function by promoting cell cycle progression, enhancing DNA replication and inhibiting apoptosis. The current findings indicate that GDF9 is involved in steroidogenesis and the growth of chicken granulosa cells; this illustrates the important role of GDF9 in the development of avian ovarian follicles.

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Conflicts of interest

The authors declare no conflicts of interest.

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