

# FGF21 Promotes Proliferation and Estradiol Synthesis in Porcine Granulosa Cells

**Yamei Hu**

Northwest A&F University: Northwest Agriculture and Forestry University

**Xiaoge Zhou**

Northwest A&F University: Northwest Agriculture and Forestry University

**Shengjie Shi**

Northwest A&F University: Northwest Agriculture and Forestry University

**Yankun Li**

Northwest A&F University: Northwest Agriculture and Forestry University

**Liang Huang**

Northwest A&F University: Northwest Agriculture and Forestry University

**Lei Gao**

Northwest A&F University: Northwest Agriculture and Forestry University

**WeiJun Pang**

Northwest A&F University: Northwest Agriculture and Forestry University

**Gongshe Yang** (✉ [gsyang@nwafu.edu.cn](mailto:gsyang@nwafu.edu.cn))

NWAFU

**Guiyan Chu**

Northwest A&F University: Northwest Agriculture and Forestry University

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## Research

**Keywords:** Estradiol, FGF21, Granulosa cells, Ovary, Pig

**Posted Date:** July 30th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-746628/v1>

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# Abstract

**Background:** The proliferation and estradiol synthesis in granulosa cells (GCs) directly promotes follicular development. Previous studies had found that FGF21 regulated the hypothalamic-pituitary-gonad axis in response to the control of fertility. However, the functions and mechanisms of FGF21 in GCs are unclear.

**Results:** Here, we found that the mRNA and protein levels of FGF21 in the ovarian tissue of high-yielding sows (*Sus scrofa*) was higher than that in low-yielding sows. Moreover, *FGF21* was predominantly expressed in porcine GCs. Additionally, ELISA assay showed estradiol was significantly increased when overexpression of *FGF21* in porcine GCs. Meanwhile, overexpressed *FGF21* up-regulated both the mRNA and protein levels of key estradiol synthesis-related genes in porcine GCs, including *StAR*, *CYP11A1* and *CYP19A1*. Correspondingly, knockdown *FGF21* inhibited estradiol levels and its synthesis-related genes expression. Besides, overexpression of *FGF21* promoted the proliferation of porcine GCs, displayed as increasing the percentage of S-phase cells in cell cycle and EdU positive cells, including cell viability, and upregulated cell cycle genes, including cell cycle protein B (Cyclin B) and protein E (Cyclin E). Correspondingly, knockdown *FGF21* in porcine GCs suppressed the cell cycle and cell viability, as well as EdU positive cell number.

**Conclusions:** These findings highlight that FGF21 is associated with the development of GCs and may be a novel underlying regulator of porcine follicular development.

## Background

Fibroblast growth factor 21 (FGF21) is a stress-inducible hormone that has important roles in regulating energy balance and glucose and lipid homeostasis[1], which is mainly expressed in liver and muscle. The family plays important roles in the endocrine-based regulation of metabolism [1–3]. Metabolism enhances supports cell proliferation and growth[4]. Overexpressing *FGF21* promotes non-small cell lung cancer growth and migration significantly[5]. It is not a unique instance, but has its counterpart. *FGF21* inhibitor suppresses the proliferation and migration of human umbilical vein endothelial cells through the eNOS/PI3K/AKT pathway[6]. Besides, FGF21 acts on adipocytes to promote the expression and secretion of CCL11 via activation of ERK1/2, which drives recruitment of eosinophils into scWAT, leading to increases in accumulation of M2 macrophages, and proliferation and commitment of adipocyte precursors into beige adipocytes[7]. These results suggest the important role of *FGF21* in cell growth and proliferation.

In mammals, it is widely known that GCs proliferation plays a vital regulatory role in deciding the fate of follicles and follicular maturation[8–10]. One of mainly functions of GCs proliferation is to secrete estrogens. Estradiol is the most active form of natural estrogens and is known to play important roles in female reproduction [11]. Estradiol derived from cholesterol. After binding to lipoprotein receptors, cholesterol is taken up by steroidogenic cells, stored, and moved to the sites of steroid synthesis[12]. The rate-limiting step in steroid production is StAR transport cholesterol from the cytosol to the inner

membrane of the mitochondrion, where the cytochrome P450 enzymes that catalyze the cleavage of the side chain of cholesterol are located. where it is converted to pregnenolone by CYP11A1 [13]. Aromatase CYP19A1 transforms testosterone into estradiol in GCs[13, 14]. Furthermore, estrogens produced in GCs are suggested to support the survival and proliferation of GCs and facilitate the maturation of follicles[15]. Studies shown that the proliferation of GCs is also regulated by cell cycle genes, including *Cyclin B*, *Cyclin E*, *Cyclin D*, *CDKs* and *p21*[16, 17].

The development of mammalian follicles is a key process in the ovary, and the proliferation and growth of GCs directly promotes follicular development [18]. As they proliferate and differentiate during follicular development, GCs can synthesize a variety of hormones and growth factors, and they express their receptors[19, 20]. These substances regulate the growth, differentiation and maturation of follicular inner membrane cells and oocytes through gap junctions, and then, they regulate follicular development[21]. Therefore, the normal functions of GCs to proliferate and secrete hormones are essential for the continuous development of follicles.

Recent studies have shown that *FGF21* regulated female reproduction through neuroendocrine pathways [22]. However, other data indicated that *FGF21* has no direct effect on female fertility in mice [23]. Interestingly, *FGF21* cooperates with adiponectin to play a beneficial role in preserving mouse primordial follicles in females fed a low-protein diet [24]. Besides, studies also have revealed that *FGF21* is associated with the cell growth and proliferation [4, 6, 25, 26]. However, no studies have focused on the *FGF21*'s expression level and biologically functions in porcine GCs. Therefore, whether and how *FGF21* affects follicular development still requires further investigation. Here, porcine ovaries were used to study the regulatory mechanism of *FGF21* in ovarian granulosa cell proliferation and hormone secretion. The results increase our understanding of *FGF21*'s effects on follicular development.

## Materials And Methods

### Ovary collection from high- and low-yielding sows (*Sus scrofa*)

Litter size records (a total of 8,567 parities) at Hanshiwei Food Co., Ltd. (Dahua pig farm, Guangxi, China) from 2016 to 2018 were collected, sorted and analyzed. The low-yielding group was defined as less than 9.3 heads per litter, and the high-yielding group was defined as more than 14.7 heads per litter [16]. The ovarian tissues of three high- and low-yielding 'Yorkshire' × 'Landrace' sows having the same physiological status and similar body weights were collected.

### Ovarian tissues and immunohistochemistry

Fresh ovarian tissues were removed, washed with normal saline, and fixed with paraformaldehyde, at greater than 7 to 10 times the volume of the ovaries, for 24 h at room temperature. They were then

embedded in paraffin and sliced into sections for HE staining. The largest cross sections were selected for FGF21 (sc-81946,1/1000) immunohistochemical staining.

## Granulosa cells isolation and culture

Fresh porcine ovaries(n=30) from cyclic sows were collected and immediately placed in a saline solution containing 100 IU/mL of double-antibody (cyano-streptomycin) at 37 °C. These ovaries were shipped back to the laboratory within 2 h and cleaned in a thermostatic saline solution. The antral follicles 3-6 mm diameters located on the ovarian surface were punctured to collect follicular fluid. The cumulus-oocyte complex was removed using a 70-mm cell strainer to obtain GCs. GCs were resuspended in DMEM/F12 (Servicebio, Wuhan, China) containing 3% BSA, and then, they were inoculated into a cell culture well and cultured in a cell incubator with 5% CO<sub>2</sub> at 37 °C [27].

## Transfection of *FGF21* siRNA and FGF21 recombinant protein treatment

To study the effects of *FGF21* on proliferation and hormone synthesis in GCs, transfection experiments were performed at a cell density of 40% to 50%. All the transfection procedures were performed with Xtreme-GENE siRNA Transfection Reagent (Roche) in accordance with the manufacturer’s instructions. In addition, GCs were treated with different FGF21 recombinant protein concentrations (0.1, 1 and 10 ng/mL). After transfection and recombinant protein exposure for 24 h, the culture supernatants and cells were collected for subsequent experiments.

## Total RNA extraction, RNA reverse transcription and RT-qPCR

Total RNAs from different cells were extracted using Trizol (TaKaRa, Otsu, Japan), and the concentrations were measured using a NanoDrop 2000 (Thermo, Waltham, MA, USA). Total RNA (500 ng) was reverse transcribed to cDNA using a reverse transcription kit (TaKaRa). Quantitative real-time (RT) -qPCR was performed on a StepOne Real-Time PCR Machine (ABI, Carlsbad, CA, USA). The relative level of mRNA was standardized to  $\beta$ -actin and calculated using the  $2^{-\Delta\Delta Ct}$  method. Primer sequences used for RT-qPCR are listed in Table 1.

Table 1

RT-qPCR primer sequences used in the study (*Sus scrofa*).

Gene Name	Forward (5'-3')	Reverse (5'-3')
FGF21	CCTGAAGCCAGGGGTCATTC	CGATCCGTACAGTCTCCCGT
StAR	CGTTTAAGCTGTGTGCTGGG	TCCATGACCCTGAGGTTGGA
3 $\beta$ -HSD	TCTTGTCTGCTTCTCGCCAC	CAACTGAGACTTGGGTGCCA
CYP11A1	GGGCAACCCATTTCTACCA	CGAGCACTGGTGGTACAGAC
CYP19A1	TCCGCAATGACTTGGGCTAC	GCCTTTTCGTCCAGTGGGAT
Cyclin B	AATCCCTTCTTGTGGTTA	CTTAGATGTGGCATACTTG
Cyclin D	TACACCGACAACCTCCATCCG	GAGGGCGGGTTGGAAATGAA
Cyclin E	AGAAGGAAAGGGATGCGAAGG	CCAAGGCTGATTGCCACACT
p21	ACGTCTCAGGAGGACCATGT	AGAAGATCAGCCGGCGTTTG
$\beta$ -actin	GGAATTTCGAGCAGGAGATGG	AGGAAGGAGGGCTGGAAGAG
SR-BI	GCTGTTCATCCCCATCGTCT	GGCCTGAATGGCCTCCTTAT
SREBP2	CTCACCTTCCTGTGCCTCTC	CCAGAAGGTGACTGAGGAGC

## Western blotting

The GCs were washed three times with PBS (pH=7.4), and then, 120  $\mu$ L RIPA (Beyotime, Shanghai, China) supplemented with protease inhibitors (Pierce, Rockford, IL, USA) was added to each well of the 6-well plate. Afterward, samples were lysed on ice for 20 min, collected and centrifuged at 12,000  $\times$ g for 10 min at 4°C. A BCA protein assay kit (Cwbio, Beijing, China) was used to determine protein concentrations. A 1/4 volume of 5 $\times$  loading buffer (Cwbio, Beijing, China) was added to an aliquot of the supernatant, and a 20- $\mu$ g protein sample was separated on a 10% SDS-polyacrylamide gel. After electrophoresis, the samples were transferred to the polyvinylidene fluoride (PVDF) membranes (CST, Boston, MA, USA), which were blocked with 5% skimmed milk powder for 2 h. The membranes were then incubated at 4°C overnight with primary antibodies (1:1000) against StAR, CYP11A1, CYP19A1, 3 $\beta$ -HSD,  $\beta$ -actin, SR-BI, SREBP2 and p21 (Abcam, Cambridge, UK), as well as against Cyclin B, Cyclin D, Cyclin E and FGF21 (Santa Cruz, CA, USA). The membranes were placed in secondary antibody (Boster, Wuhan, China) solutions and incubated at 4°C for 2 h. Finally, the signals were detected using a gel imaging system (Bio-Rad, CA, USA), and the intensity levels of the bands were measured using Image J software. All the experiments were repeated at least three times and mean values were derived.

## Measurement of estradiol and progesterone in the culture medium

The collected supernatant was centrifuged at 4°C for 10 min, and then, porcine estradiol and progesterone enzyme-linked immunoassays (ELISAs) were performed in accordance with the kit's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

## EdU imaging assay

GCs were seeded in 96-well plates at a concentration of  $2 \times 10^3$  concentration per well. After treating the cells with *FGF21* siRNA (si-*FGF21*) and recombinant protein (rFGF21) for 24 h, they were incubated with 50  $\mu$ M EdU (RiboBio, Guangzhou, China) for 2 h. The cells were washed twice with PBS, fixed in 4% paraformaldehyde for 30 min, neutralized with 2 mg/mL glycine for 5 min and permeabilized with 0.5% TritonX-100 for 10 min. Afterward, 100  $\mu$ L of 1X Apollo® staining reaction solution was added to each well, and the plates were protected from light at room temperature while on a shaker for 30 min. The reaction solutions were discarded after staining. Then, 100  $\mu$ L of penetrant (0.5% TritonX-100 in PBS) was added to each well, and the samples were washed twice on a shaker for 10 min each time. Then, the penetrant was discarded. Finally, 100  $\mu$ L 1X Hoechst 33342 was added to each well to stain the nuclei. The stained cells were observed using a Nikon TE2000 microscope (Nikon, Tokyo, Japan) and the data were analyzed using Image J software.

## Cell counting kit 8(CCK8) analysis

GCs were seeded in 96-well plates at a  $2 \times 10^3$  concentration per well. After treating the cells with *FGF21* siRNA and rFGF21 for 24 h, 10  $\mu$ L CCK8 reagent was added to each well and incubated for 3 h at 37°C. Absorbance was measured at 450 nm using Vector 5 (Waltham, MA, USA).

## Flow cytometry

GCs were seeded in 6-well plates at a  $4 \times 10^5$  concentration per well. The cells were treated with si-*FGF21* and rFGF21 for 24 h, and then, cells were digested with 0.25% trypsin and collected in 10 mL centrifuge tubes. The tubes were centrifuged at  $1,200 \times g$  for 10 min, and the supernatants were discarded. The cells were resuspended in 70% cold ethanol and placed overnight at 4°C. The cells were washed twice and stained with 50 mg/mL propidium iodide (PI) for 30 min. Finally, the cell cycles of the porcine GCs were analyzed using flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

## Statistical analysis

Each experiment was conducted at least three times and data for continuous variables are presented as mean values with standard errors of the mean (SEM). Statistical comparisons of mean values between

groups were performed using t-test and multiple comparisons were performed using a one-way analysis of variance (ANOVA). Differences were considered to be statistically significant at a  $P$ -value was  $< 0.05$ .

## Results

### FGF21 highly expression in high-yielding porcine ovaries

The *FGF21* expression levels in ovaries of high-yielding and low-yielding sows was examined. The *FGF21* mRNA and protein levels in the ovaries of high-yielding sows were significantly higher than in low-yielding sows (Fig. 1A, B). Immunohistochemical staining revealed that *FGF21* was mainly expressed in GCs (Fig. 1D). To further confirm whether *FGF21* was associated with follicular development, GCs in porcine ovarian follicles of different diameters were isolated and cultured, and the *FGF21* mRNA levels were detected. The expression of *FGF21* in GCs of large follicles (diameters  $> 6$  mm) was significantly higher than in small follicles (diameters  $< 3$  mm) (Fig. 1C). The results suggest that *FGF21* is expressed in porcine ovarian GCs.

### Knockdown FGF21 inhibites granulosa cells estradiol synthesis

During follicular development, the main function of GCs is to secrete estradiol. To investigate the role of *FGF21* in the estradiol synthesis of GCs, *FGF21* knockdown experiments were carried out in GCs. A successful *FGF21* knockdown was produced (Fig. 2A). An ELISA of the *FGF21* knockdown's medium supernatant revealed that the estradiol content significantly decreased compared with the control (Fig. 2B). This finding indicates that *FGF21* plays a role in the estradiol synthesis. To confirm this hypothesis, we detected the mRNA and protein levels of key enzymes in the estradiol synthesis process and found that StAR, CYP11A1 and CYP19A1 mRNA and protein levels were also suppressed in the *FGF21* knockdown group (Fig. 2. C-E). These data indicated that knockdown *FGF21* could inhibit the synthesis of estradiol in GCs.

### Knockdown FGF21 inhibites estradiol synthesis through granulosa cells cholesterol metabolism

Cholesterol is a precursor required for steroid hormone synthesis. Therefore, to explore the *FGF21*-associated mechanism regulating estradiol synthesis, we determined the cholesterol levels of GCs after the *FGF21* knockdown. The *FGF21* expression decreased. The estradiol content of GCs decreased significantly (Fig. 3A), and the progesterone content also showed a downward trend (Fig. 3B). At the same time, the triglycerides and cholesterol levels were also determined. There was no change in the triglyceride levels (Fig. 3C), but there was significantly decreased in total cholesterol levels (Fig. 3D). Additionally, bodipy staining was used to observe the neutral lipid droplets contents in GCs. After *FGF21*

decreased, neutral lipid droplets contents in the cells increased (Fig. 3E). In addition, the knockdown of *FGF21* caused a significant decrease in the level of the cholesterol transport-related protein SREBP2 (Fig. 3F, G). Thus, *FGF21* appears to be a potential regulator of estradiol synthesis, functioning partly through cholesterol metabolism.

## Overexpression FGF21 promotes the estradiol synthesis in granulosa cells

To further confirm the effects of *FGF21* on estradiol synthesis in GCs, we used different FGF21 recombinant protein concentrations (0, 0.1, 1 and 10 ng/mL) to treat GCs. The *FGF21* overexpression was successful (Fig. 4A). Moreover, the estradiol and progesterone contents in the supernatant of the culture medium were detected. *FGF21* overexpression significantly increased the estradiol and progesterone levels (Fig. 4B, C). This finding confirmed that *FGF21* positively regulated the estradiol content of GCs. Next, we determined the expression levels of the key estradiol synthesis-associated genes *Star*, *CYP11A1* and *CYP19A1*. FGF21 recombinant protein overexpression up-regulated these key genes at both the mRNA and protein levels (Fig. 4D-H). Consequently, we hypothesized that *FGF21* promotes estradiol synthesis in GCs.

## FGF21 promotes granulosa cells proliferation

During follicular development, GCs secrete estradiol to enhance mitosis[20]. Therefore, we speculated that *FGF21*'s promotive effects on estradiol synthesis are related to GCs proliferation. To verify this hypothesis, *FGF21* knockdown and overexpression experiments were carried out in GCs to detect indicators related to cell proliferation. The *FGF21* knockdown decreased the number of 5-ethynyl-20-deoxyuridine (EdU)-positive cells (Fig. 5A, B). The CCK8 assays showed that the cell viability was consistent with the EdU results (Fig. 5C). A flow cytometry analysis also indicated that the *FGF21* knockdown reduced the proportion of cells in S phase (Fig. 5D, E). Additionally, the *FGF21* knockdown decreased the mRNA and protein levels of the proliferation-related genes *Cyclin B* and increased the levels of the proliferation suppressor gene *p21*(Fig. 5F-H). Furthermore, *FGF21* overexpression increased the number of EdU-positive cells (Fig. 6A), the number of cells in S phase (Fig. 6C, D), and mRNA and protein levels of proliferation-related genes (Fig. 6B, E and F). Thus, *FGF21* appears to promote GCs proliferation.

## Discussion

In the ovary, the basic functional unit is the ovarian follicle, which consists of an oocyte surrounded by granulosa and theca cells that are essential for normal oocyte development and steroid hormone production[28]. GC proliferation influences steroid hormone synthesis[29, 30]. Here, we found the existence of *FGF21* in porcine GCs, which suggested that this molecule is abundantly present in ovarian

follicles. *FGF21*, as an endocrine signal, may play an important role in follicular development, including GCs proliferation and steroidogenesis.

In this study, we used high-yield (> 14.7 heads/litter) and low-yield (< 9.3 heads/litter) ovarian groups of 'Yorkshire' × 'Landrace' sows to verify that *FGF21* was expressed higher in high-yield sows (Fig. 1A-C), which indicated that *FGF21* was important for female reproduction. The ovarian immunohistochemistry of *FGF21* showed that *FGF21* was abundantly expressed in follicular GCs (Fig. 1D). In addition, we isolated and cultured porcine GCs of different diameters and detected *FGF21* expression. The *FGF21* expression level was higher in larger, compared with smaller follicular GCs (Fig. 1E). In addition, time-restricted feeding improves the reproductive functions of female mice through liver *FGF21*[31]. Furthermore, *FGF21* has beneficial effects, which implies that it is a vital positive regulatory factor of follicular development.

We speculated that *FGF21* mainly regulates the physiological functions of GCs and plays important roles in follicular development. Steroid hormone production is a major biological function of ovarian GCs. Recently, several studies also have indicated that GCs proliferation influence the synthesis of steroid hormones[16, 29, 30]. In this study, we demonstrated that *FGF21* affects estradiol synthesis in porcine GCs. The overexpression of *FGF21* increased *StAR*, *CYP11A1* and *CYP19A1* expression levels, as well as estradiol productions (Fig. 4B-H), which indicated that *FGF21* promotes porcine GCs steroidogenesis.

To further explore the mechanisms of *FGF21* in regulating estradiol synthesis in GCs, we tested the content of cholesterol, a precursor substance of estradiol synthesis. There was significantly decreased in the total cholesterol levels (Fig. 3D). However, in addition to mitochondria, steroid hormones are also synthesized in lipid droplets [32]. Bodipy was used to stain the neutral lipid droplets in GCs, but it was found that after knockdown *FGF21*, the neutral lipid droplets in the cells increased. It may be because after knockdown *FGF21* and cell metabolism slows down, resulting in an increase in lipid droplets[33]. However, a large number of lipid droplets will inhibit the synthesis of steroid hormones[34]. In addition, the *FGF21* knockdown caused a significant decrease in the protein level of the cholesterol transport-related gene *SREBP2*(Fig. 3F, G). Therefore, part of the regulatory effect of *FGF21* on granulosa cell's estradiol synthesis may be explained by its effect on cholesterol metabolism. The detailed molecular mechanism of *FGF21*'s regulation of estradiol synthesis still needs further investigation.

A single-layered primordial follicle with flat GCs does not have the ability to synthesize estradiol in vitro; however, the well-developed multilayered and antral follicle, in which the ultrastructural characteristics of the thecal and granulosa cell layers are similar to an adult ovarian follicle, synthesized estradiol in vitro[35]. Estradiol synthesis increases along with the number of GCs. The endocrine role of *FGF21* was discovered in 2005, and it acts as a novel metabolic regulator that promotes cell proliferation and growth[7, 36], However, whether *FGF21* affected GCs proliferation was unclear. Therefore, we investigated whether *FGF21* affects GCs proliferation while promoting estradiol synthesis. Our experimental results indicated that *FGF21* promoted proliferation by up-regulating the mRNA and protein levels of *Cyclin B* and decreasing the mRNA and protein levels of *p21*(Fig. 5F-H, Fig. 6E, F ).Cyclin B is a immunohistochemical

marker of proliferation[37] and p21 is an important member of the cyclin-dependent kinase inhibitor family[38]. These results were consistent with those of previous studies. For example, *FGF21* drives oligodendrocyte precursor cell proliferation [39].

## Conclusions

In summary, as shown in Fig. 7, our results showed that *FGF21* was highly expressed in porcine ovarian GCs and promoted the proliferation and estradiol synthesis in GCs. They revealed that *FGF21* has the potential to directly regulate follicular development, and they provide a theoretical basis for further research on the *FGF21*-associated regulatory mechanisms of follicular development.

## Abbreviations

DAPI: 4',6-diamidino-2-phenylindole; FGF21: fibroblast growth factor 21; StAR: steroidogenic acute regulatory protein; CYP11A1: cytochrome P450 family 11 subfamily A member 1; CYP19A1: cytochrome P450 family 19 subfamily A member 1; 3 $\beta$ -HSD: hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1; si-*FGF21*: siRNA-*FGF21*. SR-BI: scavenger receptor class B member 1; SREBP2: sterol regulatory element binding transcription factor 2. E<sub>2</sub>: estradiol; P4: progesterone;

p21: p21(RAC1) activated kinase 3. Cyclin B: cell cycle protein B; Cyclin D: cell cycle protein D; Cyclin E: cell cycle protein E;

## Declarations

## Ethics approval and consent to participate

These studies were approved by Northwest Agriculture and Forestry University Animal Research Ethics Committee (Yangling, Shaanxi, China).

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no conflict of interest.

## Availability of data and material

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

## Funding

This work is supported by grants from the National Natural Science Foundation of China (NO.31802047), the Major Project of breeding new varieties of genetically modified organisms (NO.2016ZX08006003).

## Authors' contributions

HYM and CGY conceived and designed the experiments; HYM, ZXG and SSJ performed the experiments; LYK and HL contributed reagents/materials/analysis tool; YGS managed the project; HYM wrote the manuscript; GL and PWJ modified the manuscript. The authors read and approved the final manuscript.

## Acknowledgements

We gratefully acknowledge all the teachers and students in Laboratory of Animal Fat Deposition & Muscle Development of Northwest A&F University.

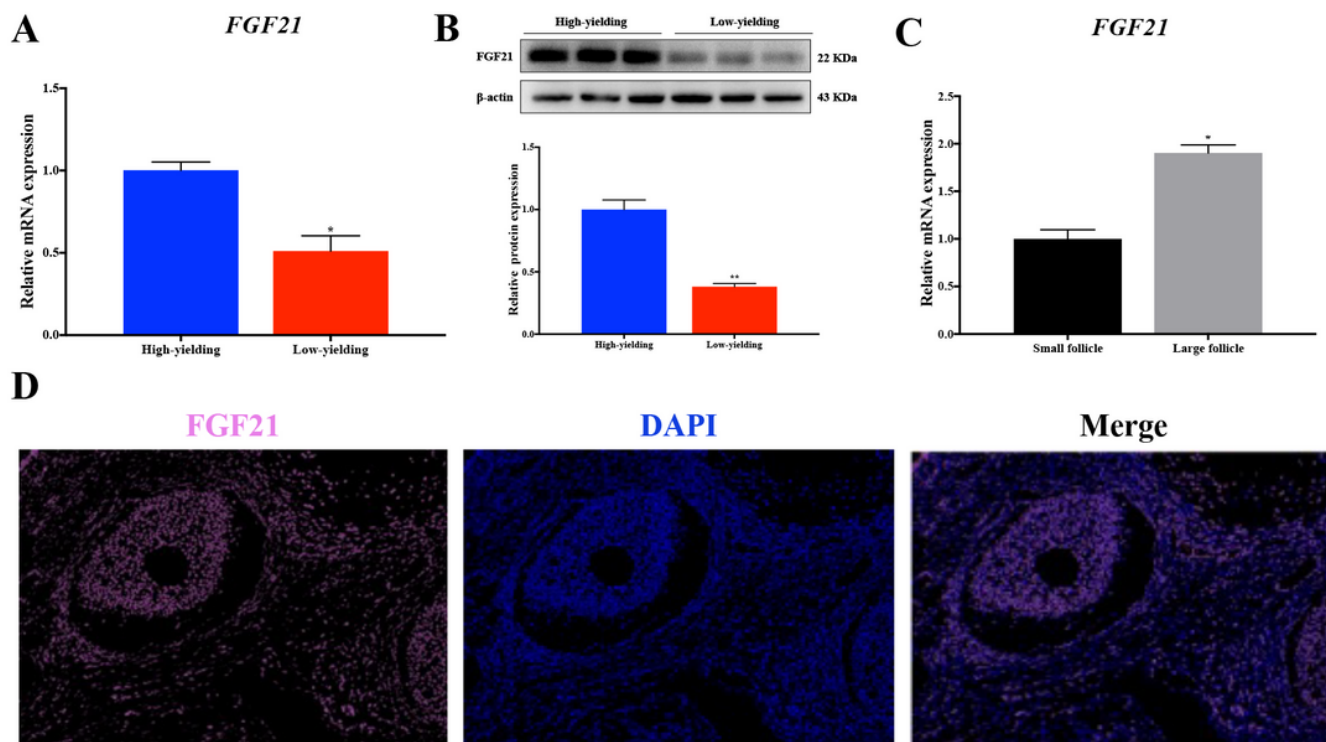
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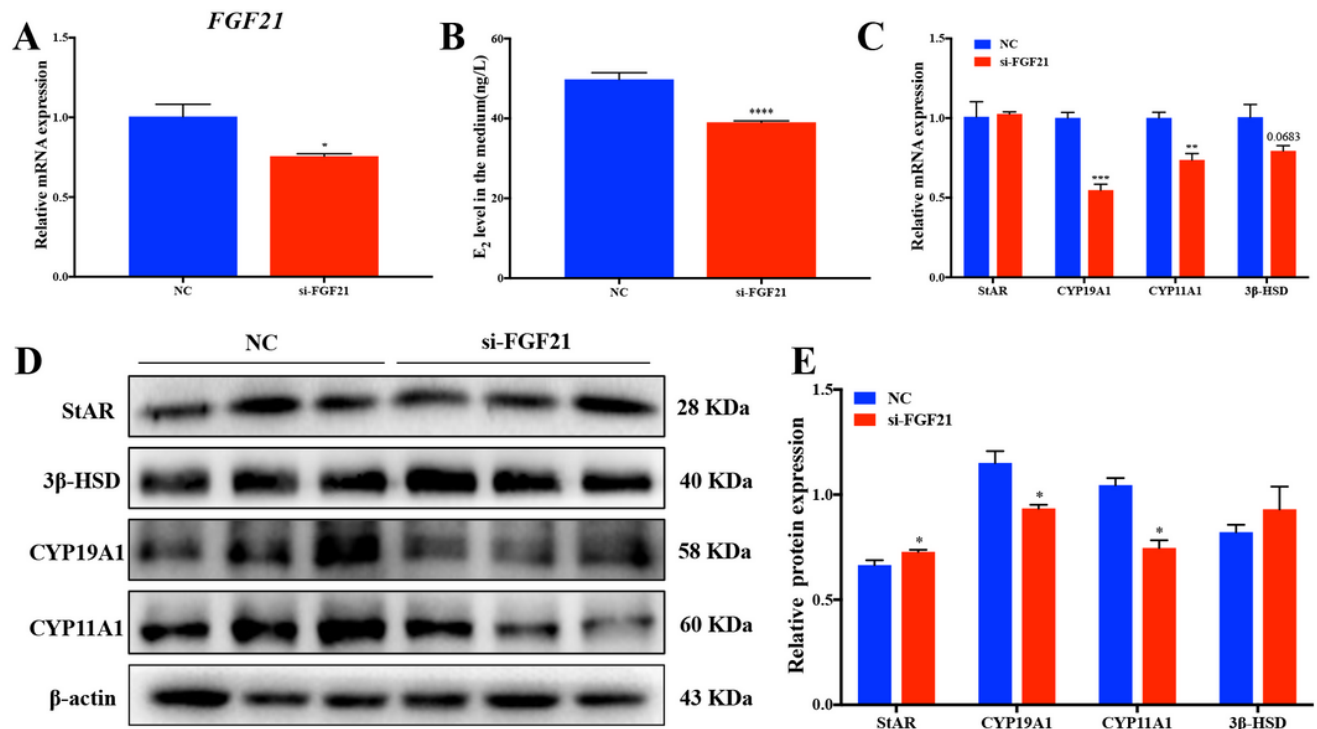
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# Figures



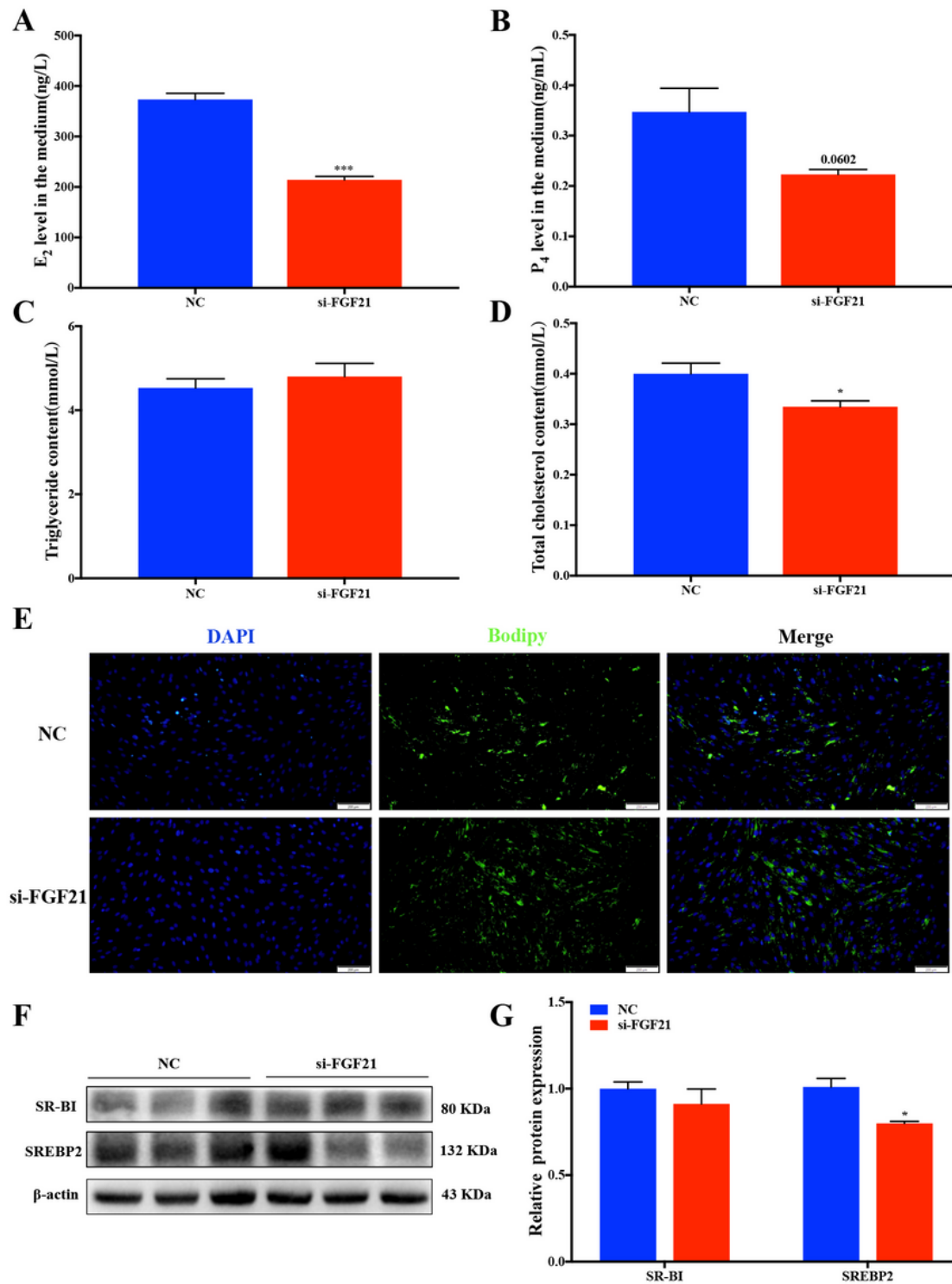
**Figure 1**

The expression of FGF21 in porcine ovary. A, mRNA levels of FGF21 in high- and low-yielding porcine ovarian (n=3). B, protein levels of FGF21 in high- and low-yielding porcine ovarian (n=3). C, FGF21 mRNA levels in GCs of large follicles (diameters > 6 mm) and GCs of small follicles (diameters < 3 mm) (n=3). D, immunohistochemical staining of FGF21 and DAPI (nuclei) on porcine ovary (n=3). Data represent the mean  $\pm$  SEM. \* p < 0.05; \*\* p < 0.01.



**Figure 2**

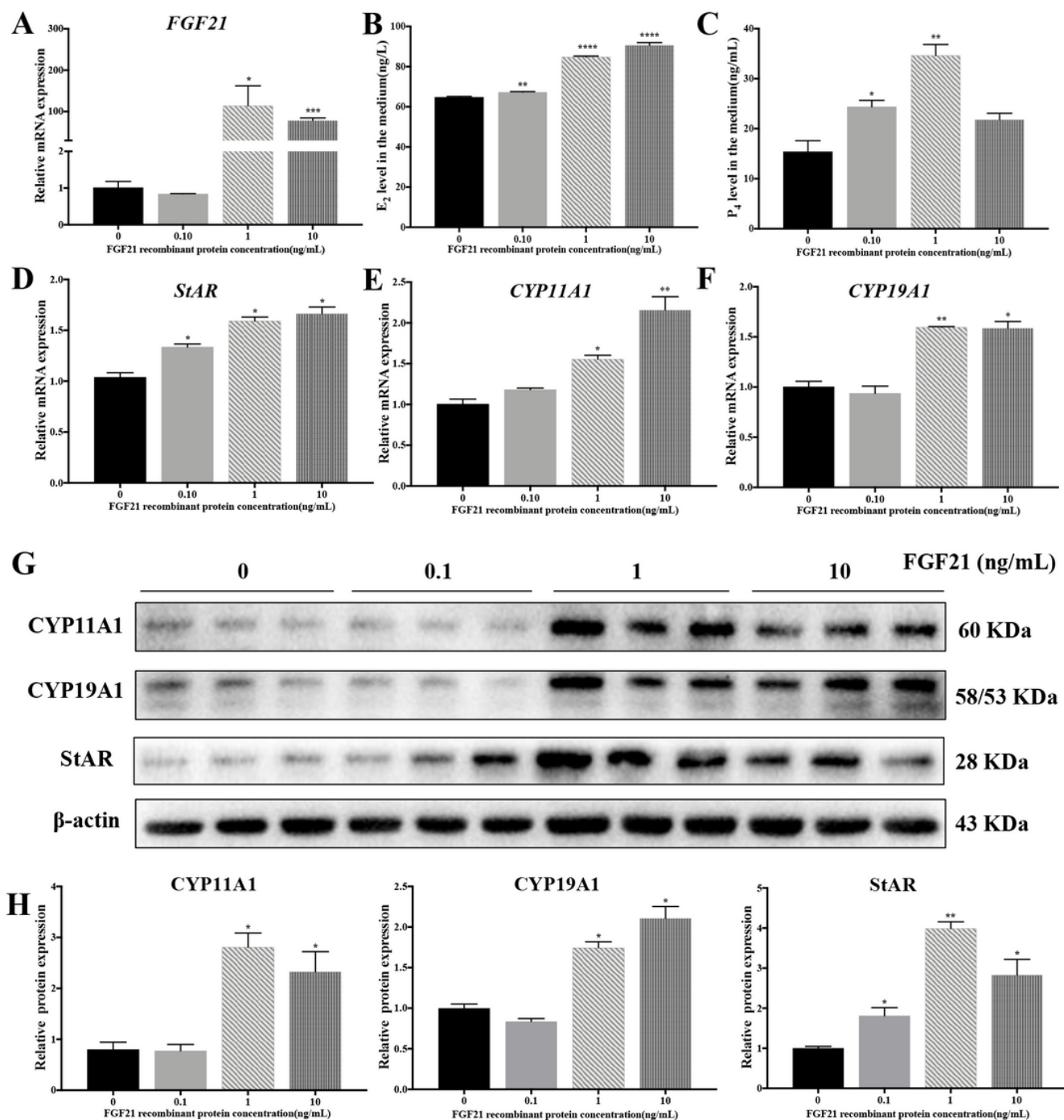
Knockdown of FGF21 inhibits the synthesis of estradiol on granulosa cells. A, knockdown efficiency of FGF21 after transfection with FGF21 siRNA compared to NC (n=3). B, estradiol concentration detected by ELISA (n=3). Culture supernatants collected 24h after FGF21 siRNA and NC treatment. C, mRNA levels of StAR, CYP19A1, CYP11A1 and 3β-HSD 24 h after knockdown of FGF21 (n=3). D, Western blot detection of StAR, CYP19A1, CYP11A1 and 3β-HSD (n=3). E, quantitation of the protein levels in D (n=3). Data represent the mean ± SEM. \* p < 0.05; \*\* p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001.



**Figure 3**

Knockdown of FGF21 inhibits the cholesterol transport. A, estradiol concentration detected by ELISA (n=3). Culture supernatants collected 24 h after FGF21 siRNA and NC treatment. B, progesterone concentration detected by ELISA (n=3). Culture supernatants collected 24 h after FGF21 siRNA and NC treatment. C, triglyceride levels detected after knockdown of FGF21 24 h (n=3). D, total cholesterol content detected after knockdown of FGF21 24 h (n=3). E, bodipy staining in GCs 24 h after knockdown of FGF21

(n=3). DAPI: nucleus, Scale bar: 200  $\mu$ m. F, Western blot detection of SR-BI and SREBP2 (n=3). E, quantitation of the protein levels in F (n=3). Data represent the mean  $\pm$  SEM.\* p <0,05; \*\* p <0.01; \*\*\*p <0.001;



**Figure 4**

Overexpression of FGF21 promotes the synthesis of estradiol on granulosa cells. A, mRNA levels of FGF21 24 h after FGF21 recombinant protein (0,0.1,1,10 ng/mL) to treat GCs (n=3). B, estradiol concentration detected by ELISA (n=3). Culture supernatants collected 24 h after FGF21 recombinant protein (0,0.1,1,10 ng/mL) treatment. C, progesterone concentration detected by ELISA (n=3). Culture

supernatants collected 24 h after FGF21 recombinant protein (0,0.1,1,10 ng/mL) treatment. D, E, F, mRNA levels of StAR, CYP19A1 and CYP11A1 24 h after overexpression of FGF21 (n=3). G, Western blot detection of StAR, CYP19A1 and CYP11A1 (n=3). H, quantitation of the protein levels in G (n=3). Data represent the mean  $\pm$  SEM.\* p <0.05; \*\* p <0.01; \*\*\*p <0.001; \*\*\*\*p <0.0001.

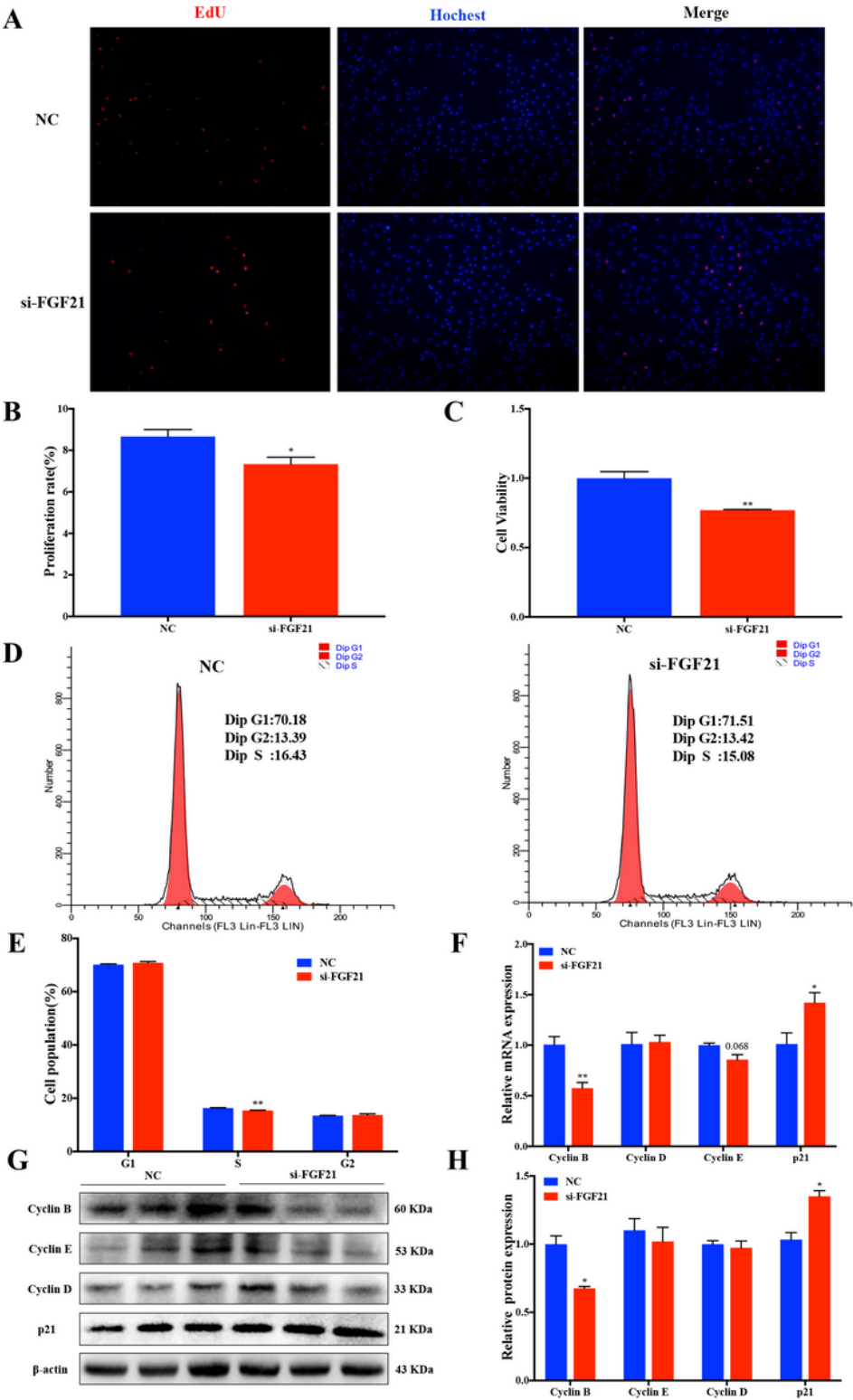
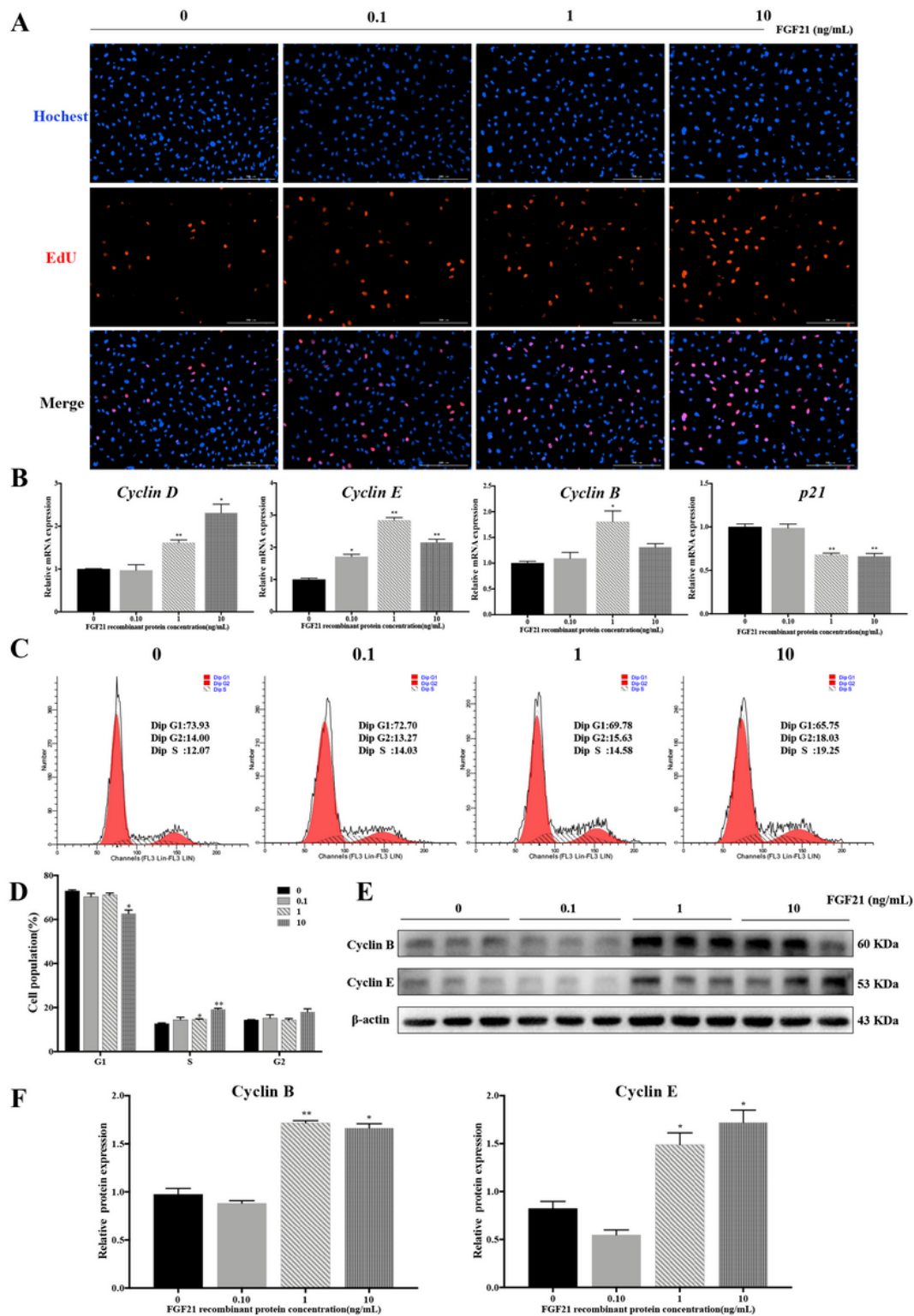


Figure 5

Knockdown of FGF21 inhibits granulosa cells proliferation. A, EdU and Hoechst (nucleus) staining analysis 24 h after knockdown of FGF21 (n=3). B, percentage of EdU-positive cells/total cells (n=3). C, CCK8 assay detects cell viability 24 h after knockdown of FGF21 as absorbance value at 450 nm after incubation with 10% CCK8 solution for 3 h. D, flow cytometry analysis 24 h after knockdown of FGF21 (n=3). E, statistical results of flow cytometry (n=3). F, mRNA levels of Cyclin B, Cyclin D, Cyclin E and p21 24 h after knockdown of FGF21 (n=3). G, Western blot detection of Cyclin B, Cyclin D, Cyclin E and p21 24 h after knockdown of FGF21 (n=3). H, quantitation of the protein levels in G (n=3). Data represent the mean  $\pm$  SEM.\* p <0,05; \*\* p <0.01;



**Figure 6**

Overexpression of FGF21 promotes granulosa cells proliferation. A, EdU and Hoechst (nucleus) staining analysis 24 h after FGF21 recombinant protein (0,0.1,1,10 ng/mL) treatment (n=3). B, mRNA levels of Cyclin B, Cyclin D, Cyclin E and p21 24 h after FGF21 recombinant protein (0,0.1,1,10 ng/mL) treatment (n=3). C, flow cytometry analysis 24 h after FGF21 recombinant protein (0,0.1,1,10 ng/mL) treatment (n=3). D, flow cytometry analysis 24 h after FGF21 recombinant protein (0,0.1,1,10 ng/mL) treatment

(n=3). E, Western blot detection of Cyclin B and Cyclin E 24 h after FGF21 recombinant protein (0,0.1,1,10 ng/mL) treatment (n=3). F, quantitation of the protein levels in E (n=3). Data represent the mean  $\pm$  SEM.\*  $p < 0,05$ ; \*\*  $p < 0.01$ .

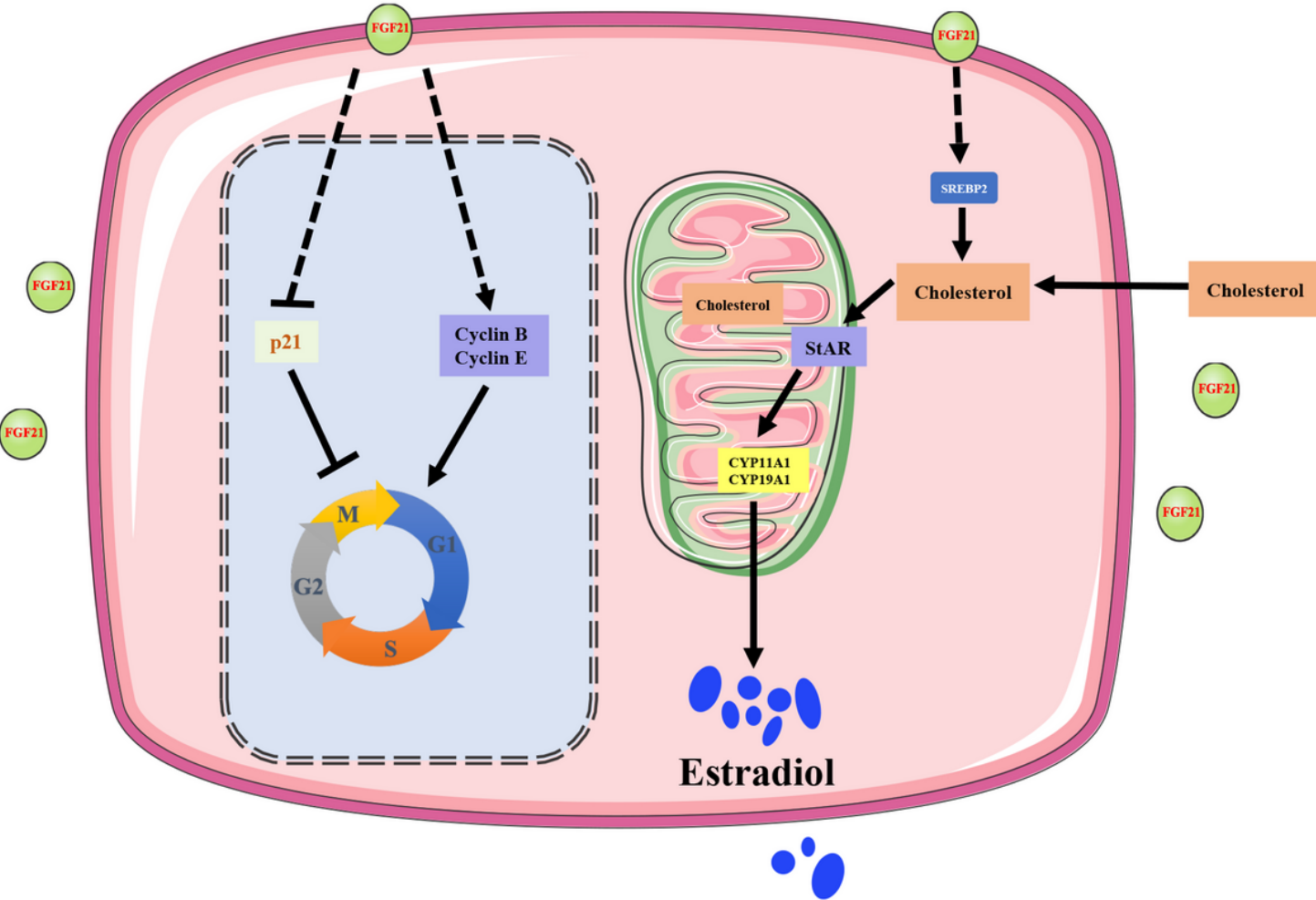


Figure 7

Schematic diagram of FGF21 regulation on porcine GCs proliferation and estradiol synthesis.