

# RAF1 as Downstream Molecule Mediates the FSH Signaling Pathway to Stimulate E2 Synthesis and Secretion in Mouse Ovarian Granulosa Cells

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## Research

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# Abstract

**Background:** V-raf-leukemia viral oncogene 1 (RAF1) kinase is the key factor in extracellular signal regulated pathway, which transmits signals to the downstream extracellular regulated protein kinases (ERK). Regulatory function of RAF1 has been proved to mediate steroid hormone synthesis, which played an essential physiological function in reproduction and development. Whether RAF1 takes part in the signaling events of gonadotropic hormones follicle-stimulating hormone (FSH) in ovarian is unknown.

**Results:** We found that RAF1 as downstream molecule mediates the FSH signaling pathway to stimulate estradiol ( $E_2$ ) synthesis and secretion in mouse ovarian granulosa cells (GCs). The expression of RAF1 is induced by FSH and the production of  $E_2$  is increased in the serum and primary ovarian GCs supernatant, the process of which is blocked by treating with RAF1 inhibitor (N-(2-Methyl-5'-morpholino-6'-(tetrahydro-2H-pyran-4-yl)oxy)-[3,3'-bipyridin]-5-yl)-3(trifluoromethyl) benzamide, RAF709). Inhibition of RAF1 activity by RAF709 decreased ERK phosphorylation, and suppressed the expression of cytochrome P450 family 19 subfamily a member 1 (CYP19A1) which is a major rate-limiting enzyme to participate in the last step of  $E_2$  biosynthesis.

**Conclusion:** Our results suggest that RAF1 play a pivotal mediating roles toward  $E_2$  production in FSH signaling pathway by inducing the phosphorylation of ERK and promoting the process of estradiol synthesis. RAF1 may be a potential and effective factor to regulate the function of the female mouse reproductive system.

## Introduction

Estrogens are mainly secreted by GCs in the ovary [1], and essential for the folliculogenesis [2], the development of secondary sex characteristics and the maintenance of female reproductive function [3, 4]. Estrogens is transformed from cholesterol, the conversion process is fairly and acutely regulated[5], involving steroidogenic acute regulatory protein (StAR)[6], cytochromes P450 and hydroxysteroid dehydrogenases[7]. Among regulatory enzymes, CYP19A1 and  $17\beta$ HSD1 are required for the final step in the biosynthesis of estrogens in ovary [8].

FSH acts as a key pituitary hormone, which can promote the follicles secrete hormones [9], initiate signaling events in ovarian by binding to its receptor (FSH-R) [10] and through complex signaling pathways [11]. It has been showed FSH stimulates the phosphorylation of the CREB [12] and histone H3 in PKA-dependent manner [13]. Besides, c-Fos, Sgk, and Inha promoter DNA are found to be increased in GCs response to FSH treatment by chromatin immunoprecipitation (CHIP) assays [13]. FSH has also been shown to be associated with ERK by stimulating a protein tyrosine phosphatase (PTP) [14]. But it is still poorly understood about the mechanisms of the FSH-stimulated ERK phosphorylation to enhance the estrogen secretion in granulosa cells.

RAF1 (also known as a C-RAF) is at approximately 68 kDa, the smallest isoform of the RAF kinase [15]. RAF1 kinase acts as a conserved signaling module transducing signals from the cell surface to the nucleus [16, 17]. Previous reports have shown that RAF1 receives extracellular signals[18] and functions in RAS pathway to transmit signals to the downstream kinases MEK and ERK [19], whereas RAF1 is activated by the binding small G proteins of the RAS family to the N-terminal region of the RAF proteins [20], and activated RAF1 subsequently activates MEK (MAP kinase kinase), MAP kinase [21], and also activates other downstream kinases and transcription factors [22]. In addition, it has been reported that RAF is a critical for steroidogenesis via ERK1/2 [23], and our recent study shows that RAF1 is a vital downstream signal in microRNA regulating process of pituitary hormone [24]. These collective data suggest that RAF1 is involved in the regulation of steroid hormone synthesis, which makes us to hypothesize whether RAF1 is involved in FSH signaling pathway to regulate E<sub>2</sub> synthesis and secretion in mouse GCs.

## Results

### RAF1 is highly expressed in mouse ovary at estrus stage and specifically located in the GCs

Firstly, we examined *Raf-1* mRNA and protein levels in the ovary at different stages in the duration of estrus cycle by Real-time quantitative PCR (RT-qPCR) and western blot (WB), and the results showed that RAF1 expressing was the highest at estrus both at gene and protein levels, significant higher ( $P < 0.05$ ) than at preoestrus, metoestrus and dioestrus stages, among which there was no significant difference (Fig. 1A, 1B). Further, we located RAF1 expression by using histochemistry and the results showed that RAF1 positive staining was observed in most ovary GCs (Fig. 1D). In addition, we did RAF1 immunofluorescence staining in the cultured GCs and the results (Fig. 1C) were similar to the histochemistry staining results in the ovary. These suggest that RAF1 is involved in estradiol synthesis in ovary GCs.

## Raf1 Participates In Regulating E Secretion

In order to identify whether RAF1 was involved in regulating the estradiol synthesis in mouse ovarian, 30 mg/kg RAF 709 (RAF1 inhibitor, dissolved in the vegetable oil) were intraperitoneally injected into the mice, while the negative control mice were intraperitoneally injected with vegetable oil only. The mice were sacrificed, up to 24 hours after injection. The ovaries were harvested for RAF1 expression measurement, and the blood samples were for global E<sub>2</sub> level detection by the RIA. The RT-qPCR and WB results showed that 24 h RAF709 treatment decreased RAF1 gene and protein levels by 32.49% and 39.76% respectively in vivo (Fig. 2E, 2F), and RIA results showed that the serum E<sub>2</sub> concentration decreased by 58.75%, compared with the controls (Fig. 2D). In addition, the cultured GCs cells were respectively treated with 0 (control), 0.5, 1, 2 and 5 nM RAF709 for 24 h, the RAF1 expressions both at gene and protein levels were assayed by using RT-PCR and WB. The results showed that 0.5, 1, 2 and 5 nM RAF709 efficiently down-regulating *Raf-1* mRNA and protein levels in the GCs (Fig. 2B, 2C), and

5 nM RAF709 decreased *Raf-1* mRNA and protein levels by 72.6% and 71.6% respectively. The RIA results showed that 24 h RAF709 treatments decreased E<sub>2</sub> concentration concentrations in the culture supernatant fluid with dose-dependence, and 5 nM RAF709 decreased E<sub>2</sub> level by 42.3%. These in vivo and in vitro results demonstrate that RAF1 has function to enhance E<sub>2</sub> synthesis and secretion in mouse ovary GCs.

### **RAF1 mediates estradiol secretion through phosphorylation of ERK in mouse ovary**

In order to confirm our hypothesis that RAF1 was involved in the FSH signaling pathway stimulating E<sub>2</sub> synthesis, the cultured GCs were respectively treated FSH, RAF 709, FSH + RAF 709, and the protein levels of FSH receptor (FSHR), RAF1, ERK-P, CYP19A1 in the culture GCs, and the E<sub>2</sub> concentration in the culture medium were respectively assayed. The results confirmed that FSH significantly increased FSHR, RAF1, ERK-P, CYP19A1 and E<sub>2</sub> levels, whereas the RAF 709 treatment blocked the enhancing effects of FSH on RAF1, ERK-P, CYP19A1 expressions and E<sub>2</sub> synthesis (Fig. 3B-E), but had no significant on FSHR compared with the controls (Fig. 3A). These data proved RAF1 act as downstream molecule to mediate the FSH signaling pathway stimulating E<sub>2</sub> synthesis and secretion in mouse primary ovarian GCs.

In order to confirm the above in vitro results in the cultured GC, the in vivo experiments were performed by injecting of exogenous FSH, RAF 709, FSH + RAF 709 in four group mice. The results showed that the levels of FSHR, RAF1, ERK-P, CYP19A1 protein expressions decreased significantly after injection of RAF709 (Fig. 4B-D), and E<sub>2</sub> synthesis and secretion showed much less than controls (Fig. 4E). However, FSHR expression were not synchronous with RAF1 protein change (Fig. 4A). These in vitro and in vivo results demonstrate that RAF1 acts as downstream molecule to mediate the FSH signaling pathway stimulating E<sub>2</sub> synthesis and secretion.

### **Model of RAF1 function in mouse ovarian GCs estradiol secretion**

By the summarization of the RAF1 inhibition experiment in vitro and in vivo, studies on the role of RAF1 in regulating estradiol secretion mechanism in pattern diagrams was illustrated.

## **Discussion**

The present study shows that the RAF1 stimulates E<sub>2</sub> synthesis and secretion via FSH signaling pathway in mouse GCs. Previous reports have shown that RAF involved in the regulation of steroid hormone synthesis via ERK1/2[23] or affected the functions of steroid hormones and their receptors [25], and its interacted with microRNA [24]. The present study demonstrated RAF1 acted as a molecule of FSH pathway to trigger the ERK phosphorylation and stimulate GCs estradiol synthesis and secretion events. Our results demonstrated that RAF1 was highly expressed in ovaries GCs during estrus, and the expression intensity and pattern of RAF1 genes was similar with estrus cycle period wave change. Besides, our in vitro and in vivo results found there might have been an estradiol link with RAF1.

We assessed the hypothesis by giving mice and cell an efficacious and selective RAF1 inhibitor RAF709, which can suppress the RAS-driven cascade reactions and block the signal transduction pathway of mitogen-activated protein kinases (MAPK) [26, 27]. Our results showed that RAF709 decreased E<sub>2</sub> concentration with dose-dependence, and 5 nM RAF709 decreased E<sub>2</sub> level by 42.3% in vitro, which agreed with previous studies that E<sub>2</sub> secretion level fell by almost half when the RAF was suppressed[28]. Many studies suggested that RAF can affect the proliferation of cell through influencing the distribution of cell cycle [29–31], and we speculated that the significant and rapid change of estradiol level was caused by GCs proliferation. Our flow cytometry analysis results showed that RAF709 did not induce the suppression of cell cycle stage, but affected on the activity of estradiol synthase, which confirmed that RAF1 could regulate the expression of CYP19A1 [32] through the ERK signaling pathways [33]. Besides, our results showed that RAF1 regulated CYP19A1 by the phosphorylation of ERK, which is consistent with the findings that RAF-ERK pathway played a vital role in mediated steroidogenesis [28].

FSH is functioning via the FSHR [34], we blocked the activity of the RAF1 in the presence of FSH to study whether it can deprive the estradiol secretions or not. In agreement with previous published studies, our results proved exogenous FSH supplement could promote E<sub>2</sub> production [35, 36]. Our results also showed RAF709 only altered RAF activity, but not blocked the binding of FSH to FSHR of rat granulosa cells, further indicated that the RAF1 acted as downstream molecule to mediate the FSH signaling pathway to stimulate E<sub>2</sub> synthesis and secretion.

RAF1 may be helpful to understand the mechanism of steroid biosynthesis, which may be an effective factor for the regulation of hormone secretion and the structure and function of the female reproductive system, especially in avoiding the risky process of gestation where the hormonal readiness is not conducive to offspring or maternal survival [37, 38].

## Conclusions

In conclusion, our data demonstrate that RAF1 acts as downstream molecule to mediate the FSH signaling pathway, through the phosphorylation on ERK to affect downstream aromatase CYP19A1 and regulate E<sub>2</sub> secretion in murine ovarian GCs (Fig. 5).

## Materials And Methods

### Animals and treatments

Adopting virgin adult wild-type female mice weighing 35-45 g (confirm they are in estrus) were used for this study. Mice were conducted on a controlled temperature (20-25°C) and humidity (60-65%) environment with a 12 h light/dark cycle, and fed with basal diet and pure water.

The experimental mice were divided into four experimental groups to study RAF1 biological regulating functions induced by FSH. Treatment groups of FSH were given single dose of FSH 10 IU/mouse and

PBS was injected as control by intraperitoneal injection (IP). RAF inhibitor RAF709 were injected to the treatment groups at 30 mg/kg [39] and corn oil was injected for the controls as the followings: (i) FSH group (FSH + corn oil, n = 4), (ii) FSH-RAF709 group (FSH + RAF709, n = 4), (iii) Control group (PBS + corn oil, n = 4), (iv) RAF709 group (PBS + RAF709, n = 4).

## Reagents and antibodies

All reagents and antibodies were commercially available. RAF709 (HY-100510, MCE);Anti-Raf1 (ab137435, Abcam); GAPDH (1:2000;Am4300,Ambion,,USA);CYP19A1 antibody (1:2000;BA3704, Boster); P-ERK antibody (1:1000; CST, MA, USA); Goat anti-Rabbit IgG (1:5,000, ZB-2301; Zhongshan, Beijing, China); ECL Western Blotting substrate (32209; Thermo Scientific, Waltham, MA); DMEM/F12 (D2906;Sigma);FBS(Gibco);streptomycin (Sigma), corn oil (Sigma).

## Isolation and culture of primary ovarian granulosa cells

Normal bred mice were given IP injection of 5 IU PMSG for 48 hours and GCs were isolated and collected by follicle puncture as previous articles described [40]. The primary ovarian GCs were incubated in DMEM/F12 containing 10% fetal bovine serum. Culturing GCs in a 5% CO<sub>2</sub> incubator with saturated humidity and a constant temperature of 37°C supplemented by 100 U/mL penicillin and 100 mg/mL streptomycin. The released cells were collected and seeded in a 6-well plate at a density of 1 × 10<sup>6</sup> cells. The cell passaging was conducted after primary GCs confluence reached 60%~70% and the culture medium was changed every two days. Under starvation GCs were used medium without FBS following the removal of the culture medium, after starvation the cells were treated with FSH or RAF709.

We divided experiment into four groups according to the treatment methods, (i) a FSH group (FSH + DMSO, n = 3) untreated GCs in the presence of induced by FSH, (ii) a FSH-RAF709 group (FSH + RAF709, n = 3) GCs were pre-treated with inhibitor before induced by FSH, (iii) a control group (PBS + DMSO, n = 3) GCs plated in normal medium without FSH induction, (iv) a RAF709 group (PBS + RAF709, n = 3) GCs were pre-treated with inhibitor in normal medium without FSH induction.

## RNA extraction and Real-time quantitative PCR (RT-qPCR) analysis

The ovary samples was dissected and extracted by liquid nitrogen grinding. Total RNA was extracted from the tissue and Trizol reagent (9109, TaKaRa Biotechnology, Dalian, China) can be directly used for GCs RNA extraction and method was provided by the manufacturer. After quantified by spectrophotometry, the purified total RNA (1 μg) was used as a template for cDNA synthesis. In the 0.5 ml centrifuge tube, add the sample RNA, supplement the appropriate amount of DEPC H<sub>2</sub>O to make the total volume up to 6 microliters, add 2 Oligo (dT) in the tube, and gently mix and centrifuge, 72 °C heating for 5 min and immediately insert the centrifuge tube into the ice bath for at least 5 min. Then add the mixture of the following reagents: MLV, dNTP, RNA safe (Promega)×42 °C incubation an hour. Primers were designed as follows. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assayed as an internal control. RT-qPCR was performed using a standard Takara SYBR Premix Ex Taq protocol on an Applied

Biosystems 7500 Real-Time PCR system (Applied Biosystems). Each sample was assayed at least three times in duplicate. GAPDH expression levels were used for data normalization. The relative abundance of genes was determined using the ABI PRISM 7500 equipped software (Applied Biosystems). The relative product levels were quantified using the  $2^{-\Delta\Delta Ct}$  method. The primers for RT-qPCR analyses were as follows:

*Raf-1.*

Forward primer- *GCTAATTGACATTGCCGACA*

Reverse primer- *TTCAACCTGCTGAGAACAC*

*Gapdh.*

Forward primer- *GGTTGTCTCCTGCGACTTCA*

Reverse primer- *GGGTGGTCCAGGGTTCTTA*

### **Western blotting (WB)**

Ovaries were lysed with RIPA buffer (C1053, Applygen, Beijing, China) containing 1 mM phenylmethanesulfonyl fluoride (PMSF; 78830, Sigma). The protein concentration of each sample was quantitated by the BCA assay reagent (HX18651, Huaxingbio, Beijing, China). Equal amounts of proteins were prepared 30 ng and loading buffer size was calculated. Samples were electrophoresed on a SDS-PAGE, 5% stacking gel at 80V 30min and 12% separating gel at 120V 45min, polyvinylidene fluoride (PVDF) membrane (IPVH00010, Millipore, Billerica, MA, USA). The membrane was soaked in methanol and the target protein were transferred to a PVDF membrane 250 mA, 1.5 h, adjust the galvanometer to constant current (mA). Dissolving nonfat dried milk 5% (wt/vol) in Tris-buffered saline (TBS) and blocked the membranes for 2 h at RT, then incubated with RAF1 antibody (1:2,000; ab137435, Abcam), CYP19A1 antibody (1:1000; Cell Signaling Technologies), P-ERK antibody (1:1000; Cell Signaling Technologies), and internal control GAPDH (1:2,000, Ambion) overnight at 4°C. Washed the membrane by TBST (0.1% Tween-20 in TBS) every ten minutes and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000, ZB-2301; Zhongshan) for 1 h at room temperature. After 30 min TBST washed, the membrane was treated by ECL Western Blotting substrate (32209; Thermo Scientific, Waltham, MA) at room temperature, from 30 sec to 20 min. to detect the signal of protein.

### **Immunohistochemistry**

Using graded ethanol for ovaries tissue paraffin section dewaxing and soaked the section in 3% H<sub>2</sub>O<sub>2</sub> (vol/vol) for 20 min to eliminate endogenous peroxidase activity. Then, microwaving samples in 0.01 M sodium citrate buffer on high power for 15 min and washed with PBS. Add 10% normal goat serum for 1 h at RT to eliminate background non-specific coloring. The sections were incubated with RAF1 antibody (1:150; HY-100510, Abcam) overnight at 4°C. Wash the slides with PBS for 3 times, and biotinylated goat

anti-rabbit IgG (1:200; 11-065-14, Jackson) was incubated for 2 h at 37°C. HRP-conjugated streptavidin (1:200; 016-030-084, Jackson) was used for incubating at RT. The diaminobenzidine (DAB) was added for color rendering—the color development degree is controlled under the microscope, the appearance of brown staining was considered a positive reaction [41]

### Immunofluorescence

When GCs grew on the glass slide and fuse to 95-100%, they are removed from the incubator and fixed in 4% paraformaldehyde for 25 min. The cells were washed with PBS for 10 minutes and permeabilized with 0.1% Triton X-100 (Sigma), and then blocked using 10% normal goat serum in TBS for 1 h at room temperature. The cells were incubated with RAF1 antibodies (1:200, diluted with 1% TBS) and put it in a wet box overnight at 4 °C. The slides of the cells were washed twice for 5 min each time by PBS, followed by incubation with the FITC-labeled goat anti-rabbit IgG (GAR-FITC, ZF-0311, Zhongshan) for 60 min. The nuclei were dyed with 4,6-diamidino-2-phenylindole (DAPI, 1:1,000, D8417; Sigma) for 15 min. Nonimmune rabbit IgG was used as a negative control. Washed the glass slide with PBS for three times to remove the excess DAPI. Drop the sealant on a clean slide and attach the cell surface of the slide to the sealant.

### Radioimmunoassay (RIA)

In experiments in which RAF1 was inhibited, blood samples were collected and centrifuged and primary ovarian granulosa cells culture supernatant fluid was collected for E<sub>2</sub> determination. The concentrations of E<sub>2</sub> in media were measured using RIA reagents provided by the Beijing North Institute Biological Technology (Beijing, China).

### Statistical analysis

All data were analyzed by GraphPad Prism 6 Software (GraphPad Software Inc., San Diego, CA, USA). Data were presented as means ± SEM. One-way analysis of variance (ANOVA) and Duncan's tests were used to analyze the main effects of treatments. *P*< 0.05 was considered as significant differences between treatments group were exist.

## Abbreviations

CYP19A1: Cytochrome P450 family 19 subfamily a polypeptide 1; CHIP: chromatin immunoprecipitation; DAB: diaminobenzidine; DAPI: 4',6-diamidino-2-phenylindole; DMEM/F-12: Dulbecco's Modified Eagle's Medium and Ham F-12; E<sub>2</sub>: estrogen; ERK: extracellular regulated protein kinases; FBS: fetal bovine serum; FSH: follicle-stimulating hormone; FSHR: FSH receptor; GAR-HRP: horseradish peroxidase-conjugated goat anti-rabbit; GAM-HRP: horseradish peroxidase-conjugated goat anti-mouse; IP: Intraperitoneal injection; MAPK: mitogen-activated protein kinases; PMSF: phenylmethanesulfonyl fluoride; PVDF: polyvinylidene fluoride; PTP: protein tyrosine phosphatase; RAF1: V-raf-leukemia viral oncogene 1; RAF709: (N-(2-Methyl-5'-morpholino-6'-((tetrahydro-2H-pyran-4-yl)oxy)-[3,3'-bipyridin]-5-

yI)-3(trifluoromethyl) benzamide; RT-qPCR: Real-time quantitative PCR; RT-PCR: Reverse transcription PCR; RIPA: radioimmunoprecipitation assay; StAR: steroidogenic acute regulatory protein; TBST: Tris-buffered saline with 0.1% Tween-20; WB: western blot

## Declarations

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### Author contributions

Sheng Cui and Kemian Gou designed research; Xuan Luo and Hui Liu performed research; Xuan Luo, Hongzhou Guo and Longjie Sun wrote the manuscript.

### Conflict of interest

The authors declare no conflicts of interest.

### Authors' information

Xuan Luo and Hui Liu contributed equally to this work.

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### Ethics approval

All animal studies were approved by the Chinese Association for Laboratory Animal Sciences and Animal Experimental Ethical Committee.

### Consent for publication

This manuscript was an original research that has not been published previously

### Availability of data and materials

We declared that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

### Competing interests

The authors report no conflicts of interest in this work.

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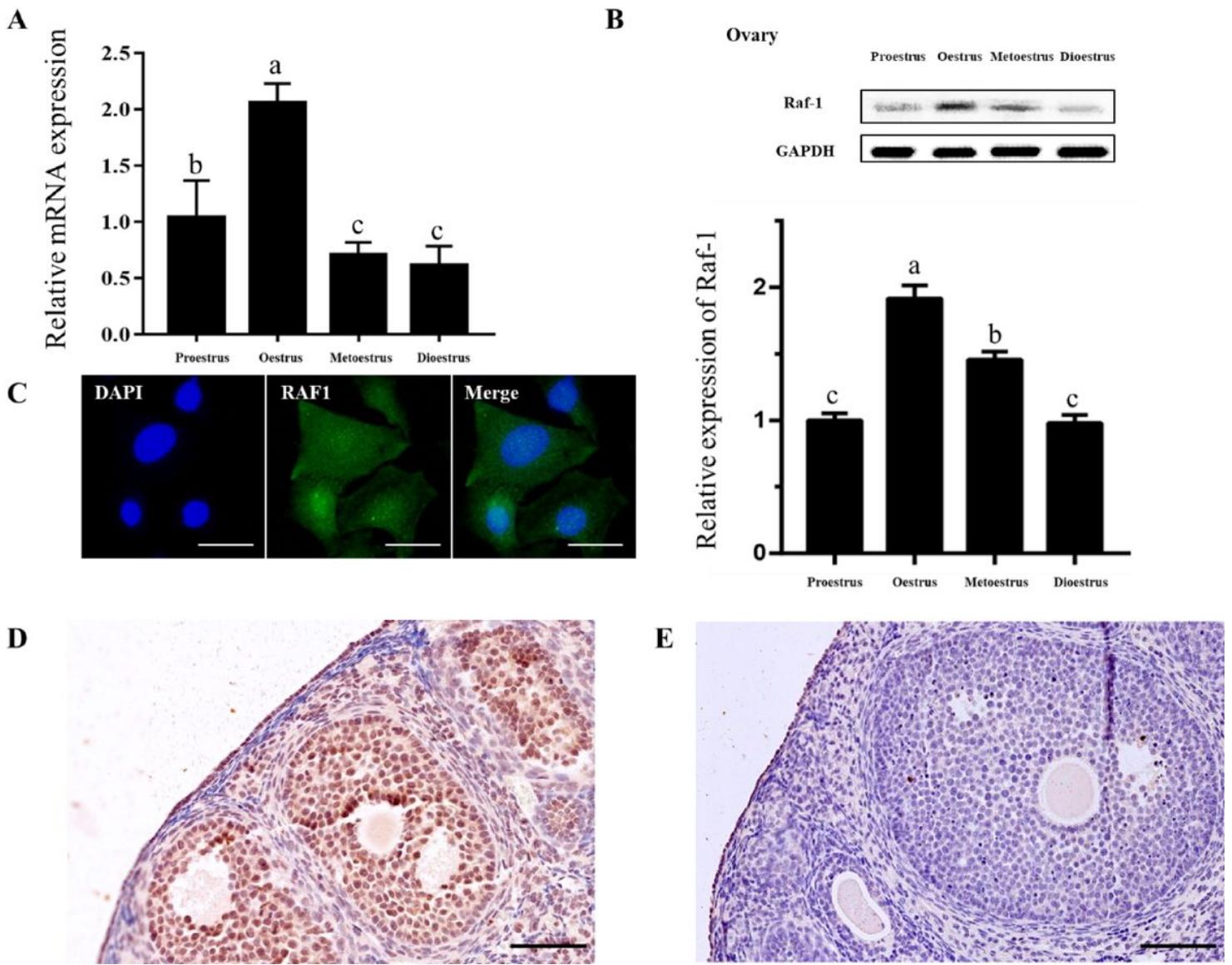
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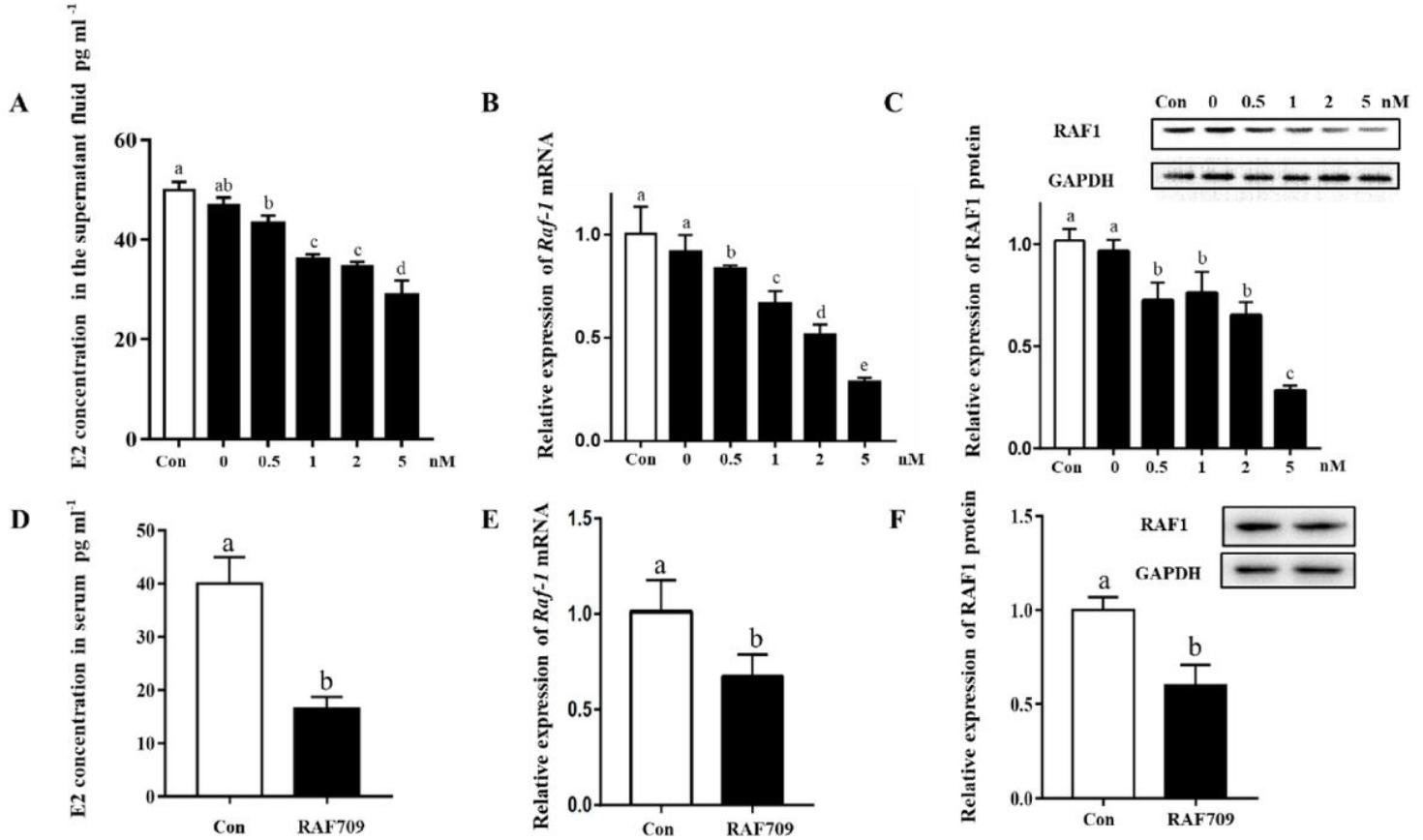
## Figures



**Figure 1**

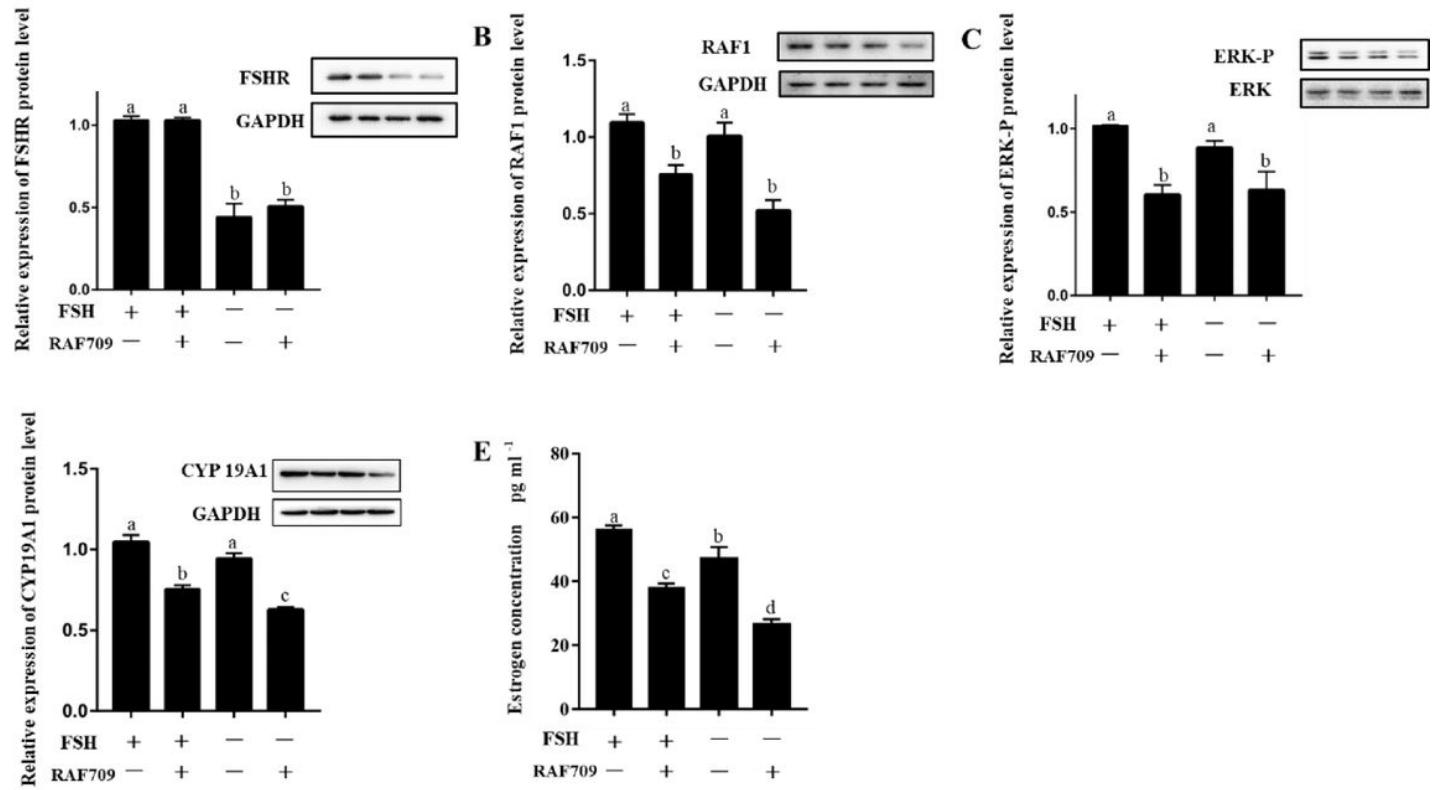
RAF1 was highly expressed in ovarian GCs of mice during estrus. A, Using RT-PCR to analyze Raf-1 mRNA expression in the ovaries during each cycle of mice. B, RAF1 protein abundances from ovary tissues during different estrus cycles were analyzed by WB with GAPDH being served as the internal reference. Different letters above error bars indicated the significant difference among results at the different estrus stage ( $P < 0.05$ ). C, Immunofluorescence technique was used to observe RAF1 location inside the primary ovarian GCs in vitro. Blue fluorescence displayed the hallmarks of the GCs nucleus by DAPI staining, bar

= 20  $\mu$ m, and green fluorescence indicates RAF1 positive signal staining. D and E, Mouse ovarian samples were examined by using antibodies against RAF1 or no primary antibodies but rabbit IgG. The localization of RAF1 proteins in ovary tissue was performed by immunohistochemical staining assay. Selectively staining was brown demonstrated RAF1 positive signals. The morphology of mouse granulosa cell was observed under high magnification microscope  $\times 100$ , bar = 50  $\mu$ m.



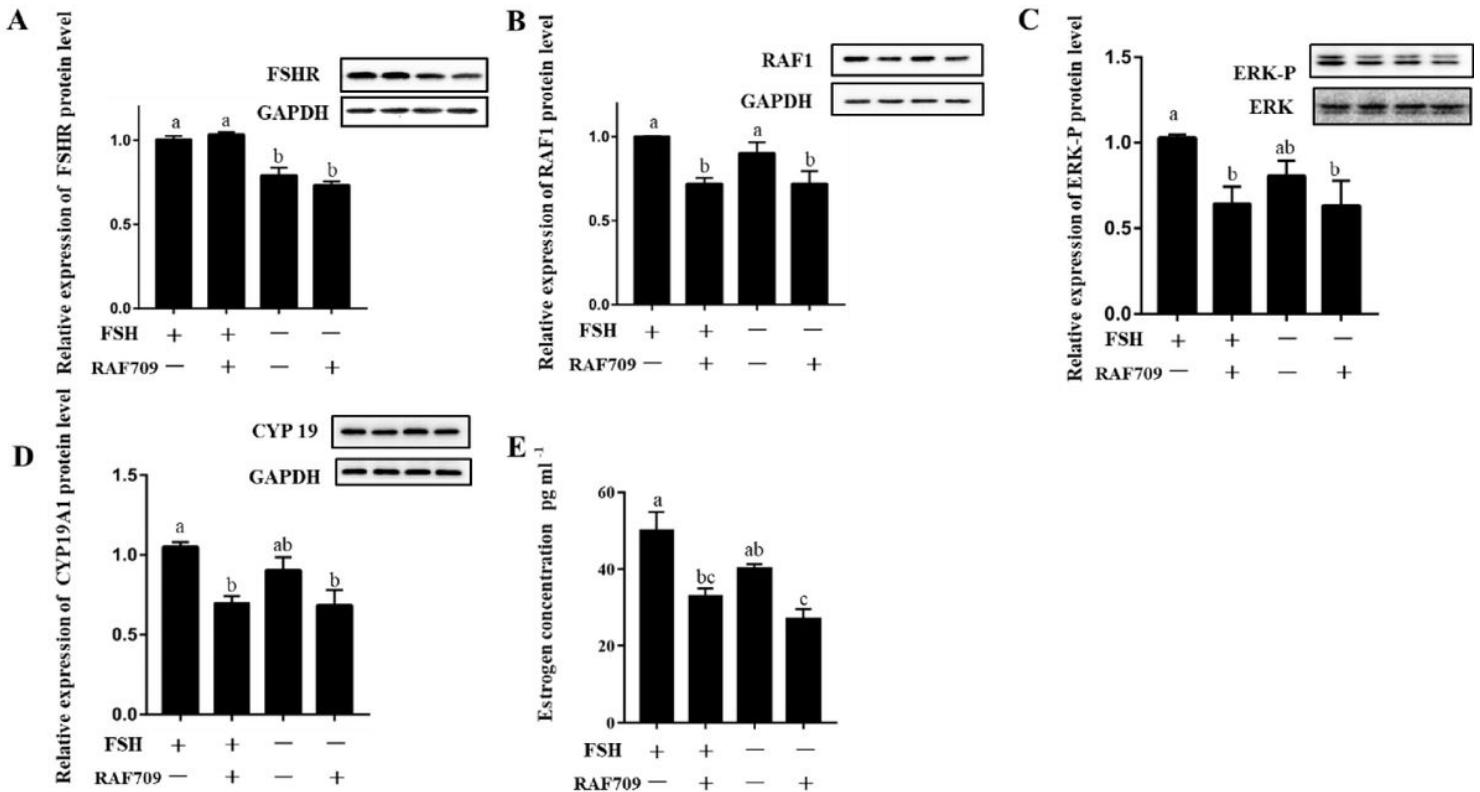
**Figure 2**

RAF1 inhibition blocked the secretion of E2. A and D, E2 concentrations in the culture media and serum. B and E, Raf-1 mRNA expression was analyzed using RT-PCR. C and F, The relative quantification of RAF1 protein expression after treated with inhibitors, and GAPDH was used as an internal control. The same letters indicate that the difference is not significant, and different letters indicate that the difference is significant ( $P < 0.05$ ). The values are the means  $\pm$  SEM of three independent experiments.



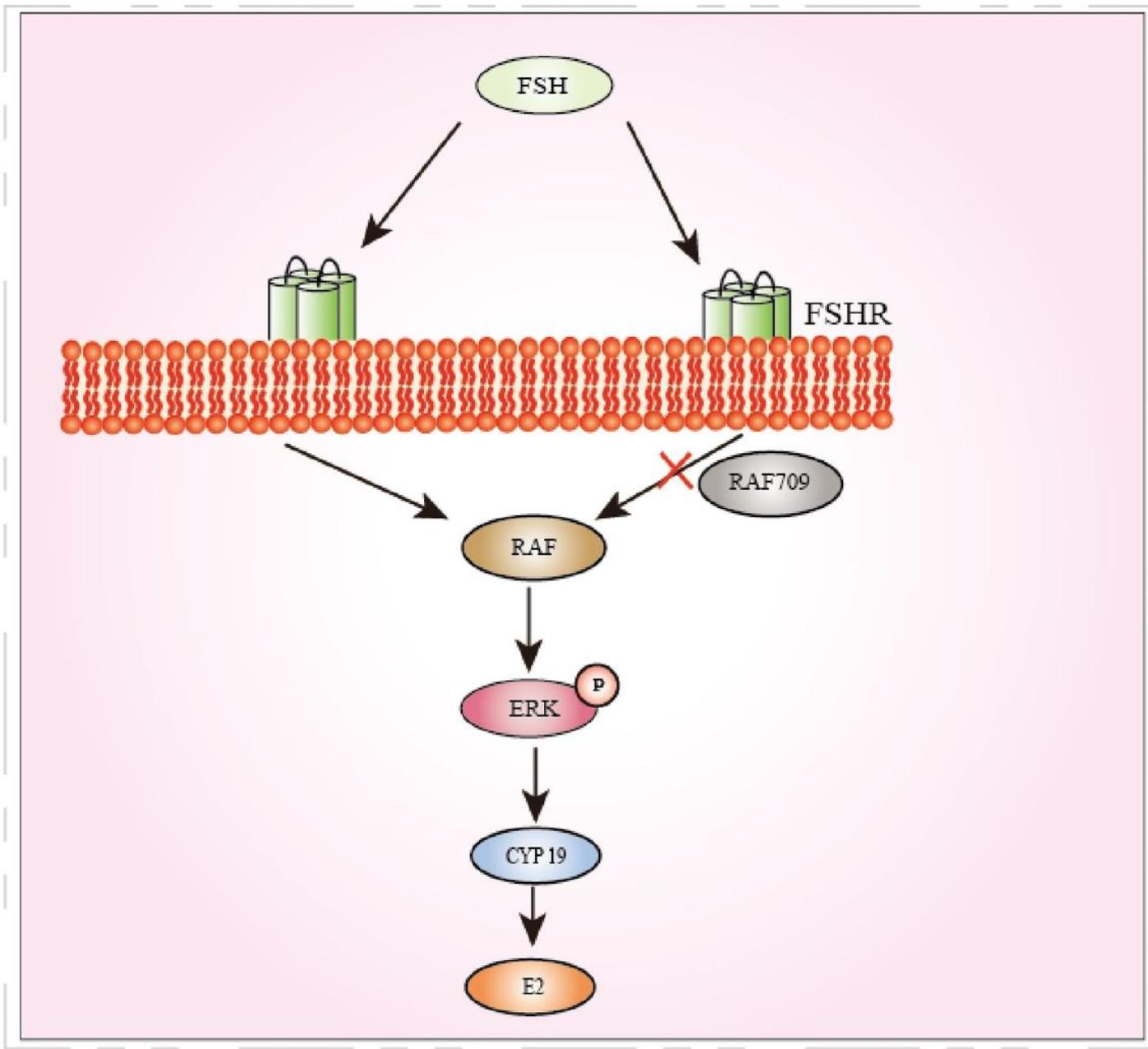
**Figure 3**

RAF1 involved in estradiol secretions by activating ERK phosphorylation. The primary ovarian GCs were treated with the 5 nM RAF709 for 24 h then induced by FSH (100 IU/L) for 24 h. A-D, The protein expression ratios of FSHR, RAF1, ERK-P and CYP19A1 were respectively detected by WB. The protein ratios were analyzed in treatment groups relative to the GAPDH protein abundance. E, Estradiol content were measured in each treatment groups by RIA. The values are the means  $\pm$  SEM of three independent experiments. Different characters indicate that there was a significant difference between the compared groups ( $P < 0.05$ ).



**Figure 4**

RAF1 act as downstream molecule to mediate the FSH signaling pathway to stimulate E2 synthesis and secretion in vivo. The mice were given single dose of FSH (10 IU/mouse) by intraperitoneal injection, and after 24 hours extending we injected the RAF709. After 24 h later blood samples of mice were collected to detect E2 content. Vegetable oils were prepared for RAF709 reference substance and PBS serve as a comparison to FSH for first injections. A-D, FSHR, RAF1, ERK-P, CYP19A1 protein expression in each treatment groups detected by WB method, the data were analyzed by Graphpad Prism version 5. E, Estradiol content in mouse serum were measured in each treatment groups by RIA. The same letters indicate that the difference is not significant, and different letters indicate that the difference is significant ( $P < 0.05$ ). The values are the means  $\pm$  SEM of three independent experiments.



**Figure 5**

RAF1 involves in the FSH signaling pathway to stimulate E2 synthesis in mouse ovary. RAF709, RAF1 inhibitor; ERK, extracellular signal-regulated kinase; CYP19A1, cytochrome P450 family 19 subfamily a polypeptide 1.