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**—Original Article—**

# miR-431 regulates granulosa cell function through the IRS2/PI3K/AKT signaling pathway

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**Abstract.** MicroRNAs (miRNAs) regulate the functions of granulosa cells by interacting with their target mRNAs. Insulin receptor substrate 2 (*IRS2*) is one of the targets of miR-431 and can be regulated by ovarian hormones. However, the role of miR-431 and the associated signal transduction pathway in ovarian development has not been studied previously. In this study, we first analyzed the expression of miR-431 and IRS2 following stimulation with pregnant mare serum gonadotropin (PMSG) during the estrous cycle or different stages of ovarian development in mice. Subsequently, we investigated the role, function, and signaling pathway of miR-431 in the human granulosa cell line, COV434. The results showed that follicle stimulating hormone (FSH) gradually decreased miR-431 levels, induced IRS2, and promoted pAKT expression. Moreover, miR-431 overexpression and IRS2 knockdown attenuated AKT activation, inhibited cell proliferation, and decreased estradiol ($E_2$) and progesterone ($P_4$) synthesis. Further, luciferase reporter assay demonstrated that *IRS2* was a direct target of miR-431. In conclusion, this study demonstrated that miR-431 regulates granulosa cell function through the IRS2/PI3K/AKT signaling pathway.

**Key words:** Cell proliferation, Estradiol, Granulosa cells, Insulin receptor substrate 2, miR-431

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Follicles, comprising of oocytes, granulosa cells, and theca cells, are the basic functional units of the mammalian ovaries. The growth and development of follicles are based on the proliferation and differentiation of granulosa cells, which are regulated by pituitary gonadotropins, including follicle stimulating hormone (FSH) and luteinizing hormone (LH), and autocrine and paracrine factors [1] such as transforming growth factor β (TGFβ), bone morphogenetic proteins (BMPs), growth and differentiation factor 9 (GDF9), and insulin-like growth factors (IGFs) [2]. Among them, FSH is an essential regulator of folliculogenesis, particularly in regulating the proliferation of granulosa cells and steroidogenesis. A previous study reported that FSH stimulates insulin receptor substrate 2 (IRS2) expression in human and rat granulosa cells [3].

IRS2 belongs to the IRS protein family that consists of four members: IRS1, IRS2, IRS3, and IRS4. The IRS proteins are the classical substrates for insulin and IGF1 receptor tyrosine kinases, which play an important role in normal physiological activities [4]. IRS2 is known to mediate metabolic homeostasis [5] and plays an important role in ovarian functions [6]. Moreover, some studies have emphasized its role in growth and proliferation [7, 8]. In addition, IRS2 can also regulate cell functions, including cell survival, steroidogenesis, and cell differentiation through the PI3K/AKT signaling pathway [6].

MicroRNAs (miRNAs) are noncoding RNAs made up of approximately 22 nucleotides, which function as post-transcriptional gene regulators and control diverse physiological and pathological processes including cell division, differentiation, migration, and apoptosis [9, 10]. Additionally, increasing number of studies have shown that miRNAs participate in the regulation of ovarian development [11–17]. A prior study revealed that IRS2 was upregulated in FSH-treated human [3, 18] and rat granulosa cells [18]. Further, some studies have suggested that miRNAs, including miR-431, can be regulated by hormones in granulosa cells [19–21]. Furthermore, a previous study reported that IRS2 is a target of miR-431 [22]. However, direct evidence to support their association with FSH-regulated granulosa cell function is lacking. The present study aimed to determine the role of miR-431 in FSH-regulated cell proliferation and $E_2$ synthesis in human granulosa cells and to elucidate the signal transduction pathways involved in this process.

## Materials and Methods

### *Chemicals*

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Stock solutions of FSH and the

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PI3K/AKT agonist, IGF1, were prepared in distilled PBS containing 0.1% BSA. A 5 mM stock solution of the AKT inhibitor, LY294002, was prepared in DMSO and stored at –20°C. The final concentrations of FSH, IGF1, and LY294002 used for culture were 50 ng/ml, 40 ng/ml, and 5 μM, respectively.

*Mice treatment and tissue collection*

Immature (3 weeks old) and adult (10 weeks old) female Kunming mice were purchased from the Laboratory Animal Central of the Jiujiang University. The mice were fed a typical diet of lab chow and housed in a single room under conditions of constant temperature (~25–28ºC, 55 ± 5% humidity, and 12-h light/dark cycle). The estrous cycles of the adult female mice were tracked by carrying out daily vaginal smears (Supplemental Fig. 1: online only) and a regular 4-day estrous cycle was used for the experiments. Immature female Kunming mice were intraperitoneally injected with 8 IU pregnant mare serum gonadotropin (PMSG; Ningbo Sansheng, Ningbo, Zhejiang, China) for different durations of time to facilitate follicular development. The ovarian cells, at different stages of the estrous cycle (diestrus, proestrus, estrus, and metestrus) and durations of time (0, 6, 12, 24, and 48 h) following PMSG stimulation, were collected and the miR-431 and IRS2 levels were analyzed. All the procedures were approved by the committee for Ethical Animal Care and Experimentation of Jiujiang University (Approval No. SYXK (GAN) 2019-0001).

*Cell culture*

COV434 cells were purchased from BeNa Culture Collection (Suzhou, Jiangsu, China). The cells were cultured in RPMI-1640 (Gibco, Grand Island, New York, USA) containing 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS, Gibco) and incubated in a humidified atmosphere with 5% $CO_2$ at 37°C.

*Lentivirus infection and screening*

miR-431, *IRS2*-siRNA, and negative control lentiviral vectors were purchased from Genechem (Shanghai, China). COV434 cells were plated in 6-well plates, cultured to 20–30% confluence, and transduced with an appropriate number of lentiviral particles (MOI = 20) in FBS-free RPMI-1640 medium supplemented with 5 μg/ml polybrene. After transduction for 12 h, the lentivirus-containing medium was replaced with fresh RPMI-1640 medium containing FBS and incubated further for 48 h. The cells were observed under a fluorescence microscope to evaluate the transduction efficiency. The efficiency and specificity of miR-431 or *IRS2*-siRNA were examined by western blotting and real-time quantitative PCR (RT-qPCR).

*Cell proliferation assay*

The cells were plated in 96-well plates at 5000 cells/well. After culturing and treatment, CCK8 was added to the cells (10 μl/well). Thereafter, the cells were incubated for 1 h at 37°C and absorbance was measured at 450 nm using a microplate reader (Bio-Rad 680). The experiments were performed in triplicate.

*Measurement of $E_2$ and $P_4$*

After 24 h of treatment, the granulosa cells were counted. The $E_2$ and $P_4$ levels in the culture supernatant were measured using an ELISA kit (Ji Yin Mei, Wuhan, Hubei, China) according to the manufacturer's instructions. The sensitivity and inter- and intra-assay coefficients of variation (CVs) of the $E_2$ and $P_4$ ELISA kit were as follows: $E_2$, 10 pg/ml, < 15%, and < 10%; $P_4$, 0.25 ng/ml, < 15%, and < 10%, respectively. Each sample was measured in triplicate.

*RT-qPCR*

Total RNA was extracted from the cells using TRIzol reagent (Life Technologies, Carlsbad, California, USA) following the manufacturer's instructions. cDNA was synthesized using the PrimeScript® RT reagent kit according to the manufacturer's instructions. Quantification of miR-431 was performed using a stem-loop RT-qPCR miRNA kit (Ribobio, Guangzhou, Guangdong, China) and small RNA U6 was used as an internal control. The primers for miR-431 and U6 were purchased from Ribobio. Endogenous β-actin was used as a reference for mRNA quantification. RT-qPCR was performed using SYBR Green qPCR SuperMix (Invitrogen, Carlsbad, CA, USA) on a 7300 Real-Time PCR System (ABI, Foster, CA, USA). All the primers used are listed in Supplemental Table 1 (online only). Each experiment was repeated independently at least three times, and the fold-change in the expression of each gene was analyzed using the $2^{-\Delta\Delta}$CT method.

*Western blotting*

Total protein was extracted using RIPA buffer supplemented with protease inhibitors. After cell lysis on ice for 30 min, the lysate was centrifuged at 12,000 rpm for 20 min, and the supernatant was stored at –80°C. Before electrophoresis, the samples were heated to 100°C for 5 min. Each sample was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto a polyvinylidene difluoride (PVDF) membrane. After incubation in blocking buffer for 1 h at 37°C, the membrane was incubated overnight at 4°C with anti-pAKT (1:1000, Cell Signaling Technology, Boston, MA, USA), anti-AKT (1:1000, Cell Signaling Technology), anti-IRS2 (1:500, Abcam, Cambridge, England) or β-actin (1:2000, Abcam) antibodies as a loading control. After washing, the membranes were incubated with secondary antibody conjugated to horseradish peroxidase at 37°C for 30 min. Finally, the immunoreactive bands were visualized using a Super Signal West Pico Trial Kit (Pierce, Rockford, Illinois, USA) according to the manufacturer's instructions.

*miRNA target gene prediction*

Three target prediction algorithms: TargetScan 6.2 (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/), and miRBase 21 (http://www.mirbase.org/), were used to search for the potential target genes of miR-431.

*Luciferase reporter assay*

Luciferase reporter assays were carried out using the Dual-Luciferase Reporter Assay System (pGL3 vector; Promega, Madison, WI, USA). A fragment of the IRS2 3′ UTR containing the wild type or mutated predicted binding site for miR-431 was inserted into the pGL3 vector, downstream of the luciferase gene, to generate the recombinant vectors pGL3-wild-type (WT) and pGL3-mutant (MUT). All constructs were verified by DNA sequencing. The pGL3 vector

containing WT- or MUT-IRS2 was transfected into COV434 cells with or without a synthetic miR-431 mimic. Luciferase activity was detected 48 h post-transfection, using the Dual-Luciferase Reporter Assay System according to the manufacturer's protocol. Data were normalized to Renilla luminescence and presented relative to the control miRNA transfected group.

*Statistical analyses*

All experimental data were analyzed using one-way ANOVA. The experimental data were analyzed using the SPSS software (Version 19.0; SPSS, Chicago, IL, USA) with Tukey's post hoc test. $P < 0.05$ was considered as statistically significant. All data are represented as the mean ± SEM, from at least three separate experiments.

## Results

*Expression of IRS2 and miR-431 at estrous cycle or different stages of ovarian development after being stimulated with PMSG in mice ovary*

To test the underlying relationship between IRS2 and miR-431 in follicular development, we examined the expression of IRS2 and miR-431 at estrous cycle or different stages of ovarian development following PMSG stimulation. The results indicated that IRS2 expression level was at its highest at estrus and lowest at diestrus (Fig. 1A and B), whereas miR-431 expression was at its highest at diestrus and lowest at estrus (Fig. 1C). In addition, miR-431 levels gradually decreased while IRS2 levels increased following PMSG stimulation (Fig. 1D–F). These results suggested a possible relationship between miR-431 and IRS2 during follicular development.

*Effect of FSH on the expression of IRS2 and miR-431, and the activation of AKT in COV434 cells*

To further test the potential relationship between IRS2, miR-431, and AKT in granulosa cells, we examined the effect of different durations (0, 3, 6, 12, 24 and 48 h) of FSH stimulation on IRS2 and miR-431 expression, and AKT activation in COV434 cells. A negative correlation between miR-431 and IRS2/pAKT expression was confirmed (Fig. 2). The expression of miR-431 gradually decreased following FSH stimulation, suggesting that miR-431 is regulated by the hormone in COV434 cells (Fig. 2A). Moreover, the increasing levels of IRS2 and pAKT indicated that the IRS2/AKT signaling pathway was induced by FSH in COV434 cells (Fig. 2B, C, and D). These data indicated that a possible regulatory relationship exists between miR-431 and IRS2/AKT signaling pathway in FSH-induced COV434 cells.

*IRS2 knockdown attenuates AKT activation, inhibits cell proliferation, and decreases E2 synthesis in FSH-induced COV434 cells*

To determine role of IRS2 in granulosa cell function, IRS2-knockdown and negative control COV434 cells were treated with FSH for 24 h. RT-qPCR and western blotting results showed that IRS2 and pAKT were efficiently repressed by IRS2-siRNA (Fig. 3A and B). CCK8 results showed that IRS2 knockdown lowered cell activity compared to the negative control (Fig. 3C). Moreover, IRS2 knockdown decreased the mRNA expression of cell cycle factors (Cyclin A1, Cyclin B1, and Cyclin D2) (Fig. 3D) and inhibited $E_2$ synthesis compared to the negative control (Fig. 3E). Further, IRS2 knockdown impaired the key genes encoding steroidogenic enzymes, including *Star*, *Cyp11a1*, and *Cyp19a1* (Fig. 3F). These results indicated that IRS2 is involved in FSH-induced cell proliferation and E2 synthesis in granulosa cells.

*miR-431 attenuates AKT activation, inhibits cell proliferation, and decreases E2 synthesis in FSH-induced COV434 cells*

To determine the effects of miR-431 on granulosa cell function, miR-431-overexpressing and negative control COV434 cells were treated with FSH. RT-qPCR results showed an increased miR-431 expression in the miR-431-overexpressing group. miR-431 was shown to impair AKT activation and IRS2 expression compared to the negative control (Fig. 4A and B). CCK8 and RT-qPCR results also showed that miR-431 inhibited cell activity and decreased the expression of Cyclin A1, Cyclin B1, and Cyclin D2 compared to the negative control (Fig. 4C and D). ELISA and RT-qPCR results revealed that miR-431 impaired $E_2$ synthesis and weakened the expression of *Star*, *Cyp11a1*, and *Cyp19a1* (Fig. 4E and F). These results indicated that miR-431 impairs FSH-induced cell proliferation and $E_2$ synthesis in granulosa cells.

*IRS2 is a target of miR-431*

To further analyze the relationship between miR-431 and IRS2, the conserved target genes of miR-431 were searched and predicted, and the expression of IRS2 was examined in the miR-431-overexpressing and negative control COV434 cells. RT-qPCR and western blotting results showed that the expression level of IRS2 was decreased in the miR-431-overexpressing group (Fig. 5A, B and C). According to TargetScan analysis, the miR-431-targeting site was in the 3′-UTR of IRS2 (Fig. 5D). To validate the miR-431 target site, a standard luciferase reporter assay was conducted. Results showed that Renilla luciferase activity for WT-transfected cells decreased about 75% in miR-431-cotransfected cells compared to that in miR-NTC-cotransfected cells (Fig. 5E). In addition, site-directed mutagenesis of the miR-431 target site abolished the inhibitory effect of miR-431 (Fig. 5E). These results indicated that *IRS2* is a direct target of miR-431.

*AKT is associated with cell proliferation and hormone secretion*

The previous results suggest that both IRS2 and miR-431 are associated with AKT activation, cell proliferation, and $E_2$ synthesis in COV434. To further investigate the role of AKT activity in FSH-induced cell proliferation and $E_2$ synthesis, an AKT inhibitor, LY294002, was added to the culture medium along with FSH and an AKT-agonist, IGF1. The results showed that LY294002 clearly inhibited FSH and IGF1-stimulated pAKT and cell activity as compared to the corresponding control group (Fig. 6A and B). In addition, LY294002 lowered $E_2$ synthesis, $P_4$ production, and the expression of cell cycle factors (Fig. 6C, D, and E). Moreover, the expression of *Star*, *Cyp11a1*, and *Cyp19a1* induced by FSH and IGF1 were downregulated by LY294002 (Fig. 6F). These results suggested that PI3K/AKT signaling pathway is involved in FSH-induced cell proliferation and hormone secretion in human granulosa cells.

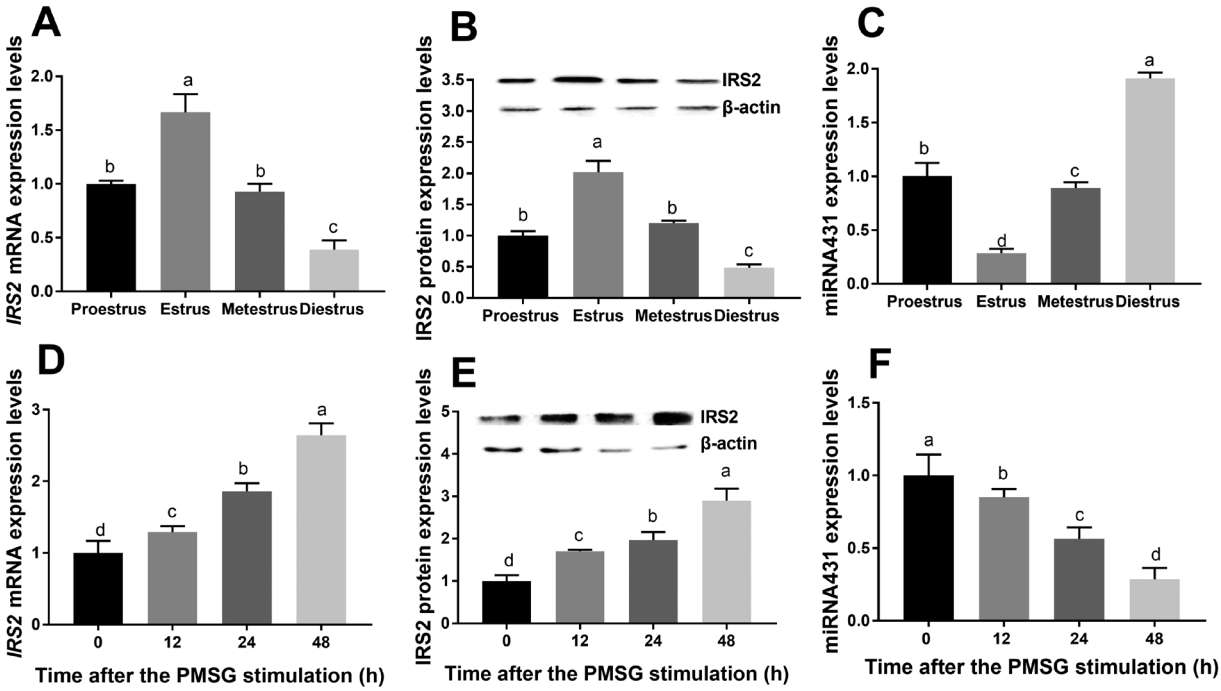


**Fig. 1.** Expression of insulin receptor substrate 2 (IRS2) and miR-431 at estrous cycle or different stages of ovarian development following pregnant mare serum gonadotropin (PMSG) stimulation of mice ovary. (A and B) The expression of IRS2 at estrous cycle. (C) The expression of miR-431 at estrous cycle. (D and E) The expression of IRS2 following PMSG stimulation for different durations of time. (F) The expression of miR-431 following PMSG stimulation for different durations of time. The results are presented as the mean±SEM and are representative of three independent experiments. The letters represent significant differences ($P<0.05$).

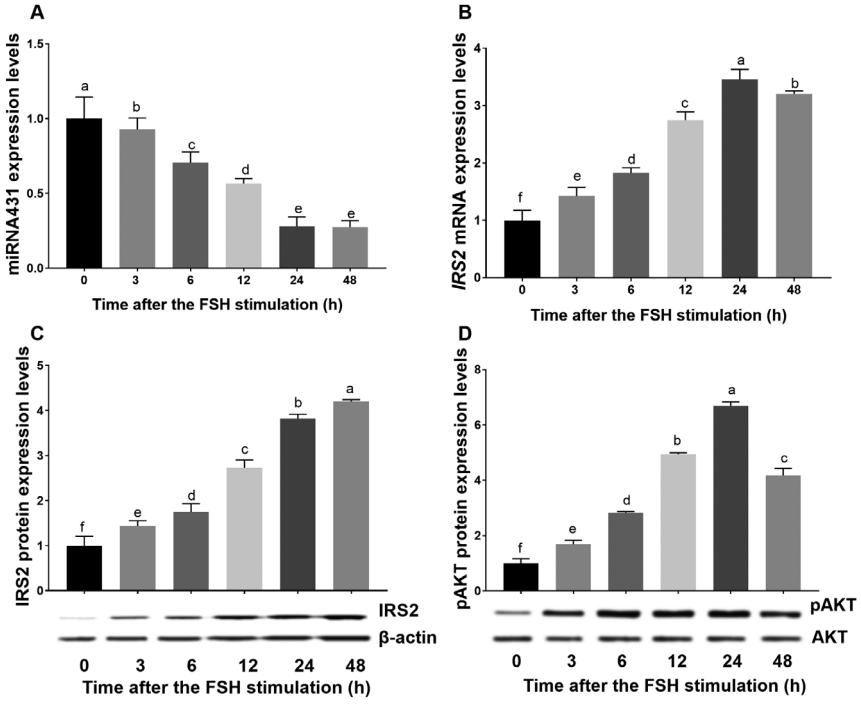


**Fig. 2.** Effects of follicle stimulating hormone (FSH) on the expression of insulin receptor substrate 2 (IRS2), miR-431, and pAKT in COV434 cells. (A) Effects of FSH stimulation on the expression of miR-431 in COV434 cells at different time points. (B and C) Effects of FSH stimulation on the mRNA and protein expression of IRS2 at different time points. (D) Effects of FSH on pAKT in COV434 cells at different time points. The results are presented as the mean±SEM and are representative of three independent experiments. The letters represent significant differences ($P<0.05$).

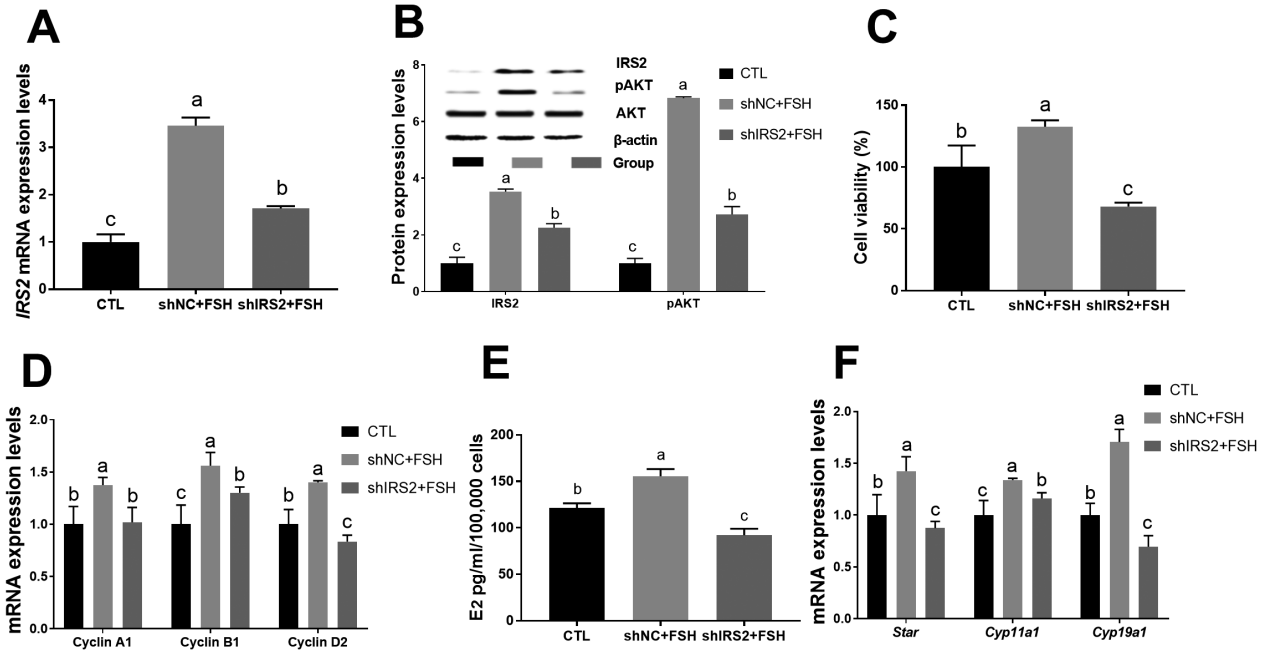


**Fig. 3.** Effects of insulin receptor substrate 2 (IRS2) knockdown on AKT activation, cell proliferation and $E_2$ synthesis in follicle stimulating hormone (FSH)-induced COV434 cells. (A) The mRNA levels of IRS2. (B) Protein levels of pAKT and IRS2. (C) Cell viability was measured using CCK8 assay. (D) The mRNA levels of Cyclin A1, Cyclin B1 and Cyclin D2. (E) $E_2$ levels in the culture medium. (F) The mRNA levels of *Star*, *Cyp19a1* and *Cyp11a1*. IRS2 and pAKT protein expression was normalized to β-actin and AKT, respectively. The data are presented as mean ± SEM of three independent experiments. The letters represent significant differences ($P<0.05$).

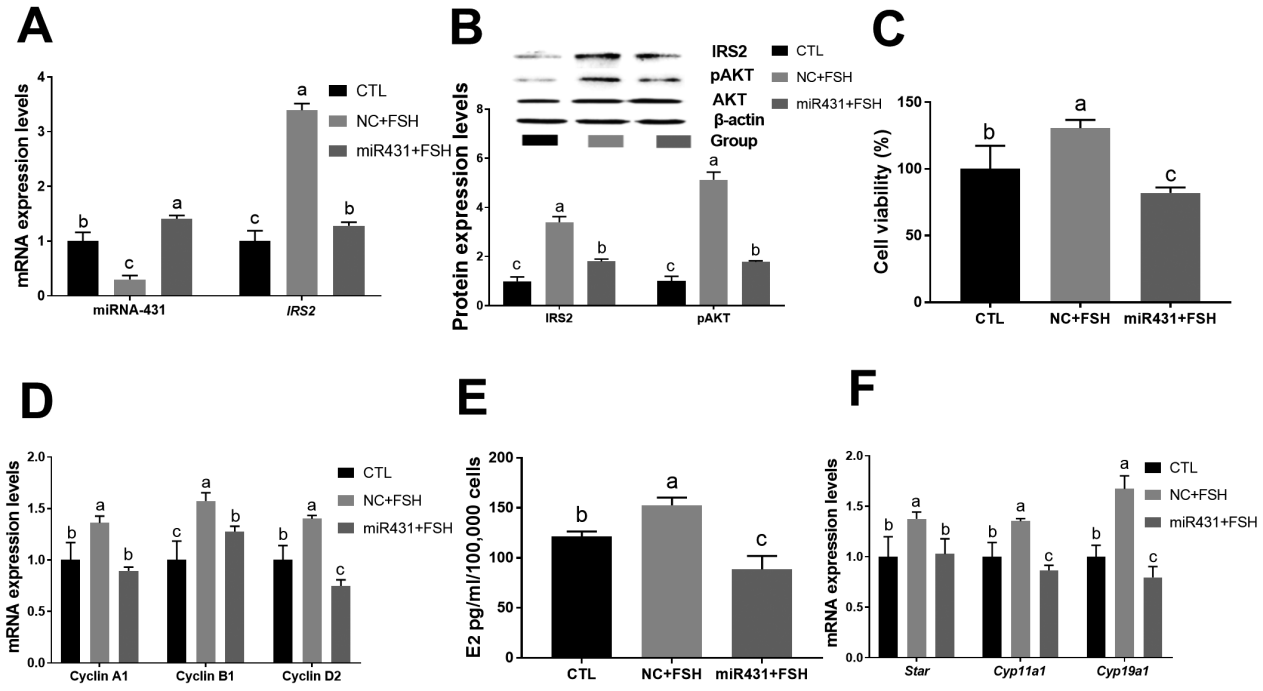


**Fig. 4.** Effects miR-431 on AKT activation, cell proliferation and $E_2$ synthesis in follicle stimulating hormone (FSH)-induced COV434 cells. (A) The mRNA levels of miR-431 and insulin receptor substrate 2 (IRS2). (B) Protein expression levels of pAKT and IRS2. (C) Cell viability was measured using CCK8 assay. (D) The mRNA levels of Cyclin A1, Cyclin B1, and Cyclin D2. (E) $E_2$ levels in the culture medium. (F) The mRNA levels of *Star*, *Cyp19a1*, and *Cyp11a1*. Protein expression of IRS2 and pAKT were normalized to β-actin and AKT, respectively. The data are presented as mean ± SEM of three independent experiments. The letters represent significant differences ($P<0.05$).

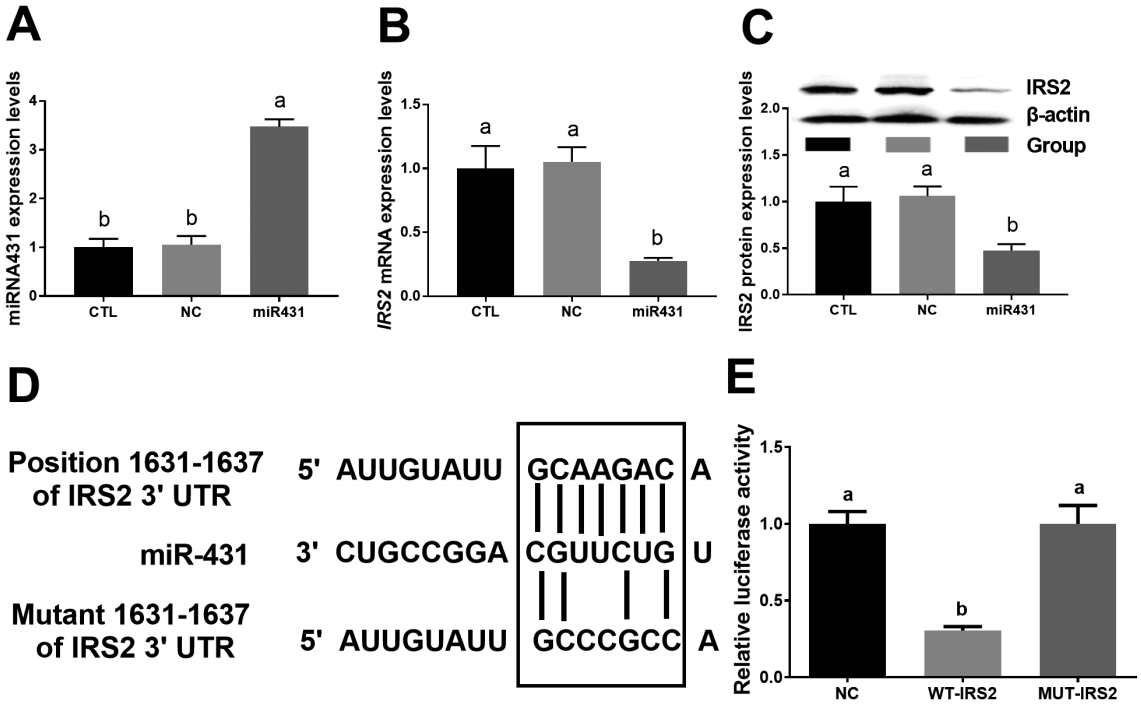


**Fig. 5.** miR-431 targets the 3′ UTR of insulin receptor substrate 2 (IRS2). (A) The mRNA levels of miR-431. (B) The mRNA levels of *IRS2*. (C) Protein levels of IRS2. (D) miR-431 target site in the IRS2 3' UTR. (E) Luciferase activity assay. *IRS2* expression was normalized to β-actin. The data are presented as mean ± SEM of three independent experiments. The letters represent significant differences ($P<0.05$).

*IGF1 rescues miR-431 overexpression- and IRS2 knockdown-induced pAKT decrease, cell growth inhibition, and $E_2$ decrease*

To further explore the relationship between IRS2, AKT, and miR-431 in FSH-induced cell proliferation and $E_2$ synthesis, miR-431-overexpressing and IRS2-knockdown COV434 cells were treated with FSH and IGF1. The results showed that both FSH and IGF1 promoted IRS2 and pAKT expression and FSH also induced miR-431 decrease, unlike IGF1 (Fig. 7A and B). Moreover, IGF1 rescued miR-431 overexpression- and IRS2 knockdown-induced AKT inhibition, cell growth inhibition, and $E_2$ decrease (Fig. 7B, C and D). We also found that IGF1 enhanced cell proliferation and $E_2$ synthesis (Fig. 7C and D).

## Discussion

Although much progress has been made in the genetic dissection of the biological networks involved in follicular development in the human ovary, the gene regulation mechanisms are still poorly understood. miRNA, as an endogenous post-transcriptional gene regulator, plays an essential role in regulating tissue growth and differentiation in mammalian animals. However, compared to the other body tissues, little is known about the functional involvement of miRNAs in the ovary. In the present study, we assessed the function of miR-431 in cell proliferation, $E_2$ synthesis, $P_4$ production, and signal transduction in granulosa cells.

We first analyzed the relationship between miR-431 and IRS2 during the estrous cycle or different stages of ovarian development following PMSG stimulation in mice. We found that the expression of miR-431 and IRS2 were negatively correlated with the estrus cycle following PMSG administration in the ovary. Subsequently, we found that miR-431 expression was downregulated and the levels of IRS2 and pAKT were upregulated after stimulation with FSH for different durations in human granulosa cell line. These results supported the possible relationship between miR-431, IRS2, and pAKT in the regulation of granulosa cell function and follicular development. Our results were similar to the results of a previous study which showed that the expression of miR-431 decreased in rat ovary following human chorionic gonadotropin administration [21]. In addition, our research further concluded that FSH can activate IRS2 and AKT signaling pathway in granulosa cells [3, 23].

Mice lacking IRS2 have small, anovulatory ovaries with reduced number of follicles [7]. Here, we found that IRS2 knockdown inhibited cell proliferation which may be due to the decrease in the expression of cell cycle factors such as the cyclins. Cyclin A1 plays a key role in cell cycle regulation by binding to Cdk2 to complete the S phase, and by binding to Cdk1 for entry into M the phase. Cyclin B1 is also involved in the mitotic process as an activator of Cdk1 [24]. Together, the downregulation of Cyclin A1 and Cyclin B1 mRNAs could lead to S phase arrest by stopping granulosa cells from entering the G2/M phase [25]. Moreover, the inhibition of IRS2/AKT resulted in a direct decrease in cyclin D2 in granulosa cells [26], which could further impair granulosa cell proliferation and follicular growth.

$E_2$ plays a key role in ovarian follicular development and normal female reproduction. In the current study, we observed a significantly

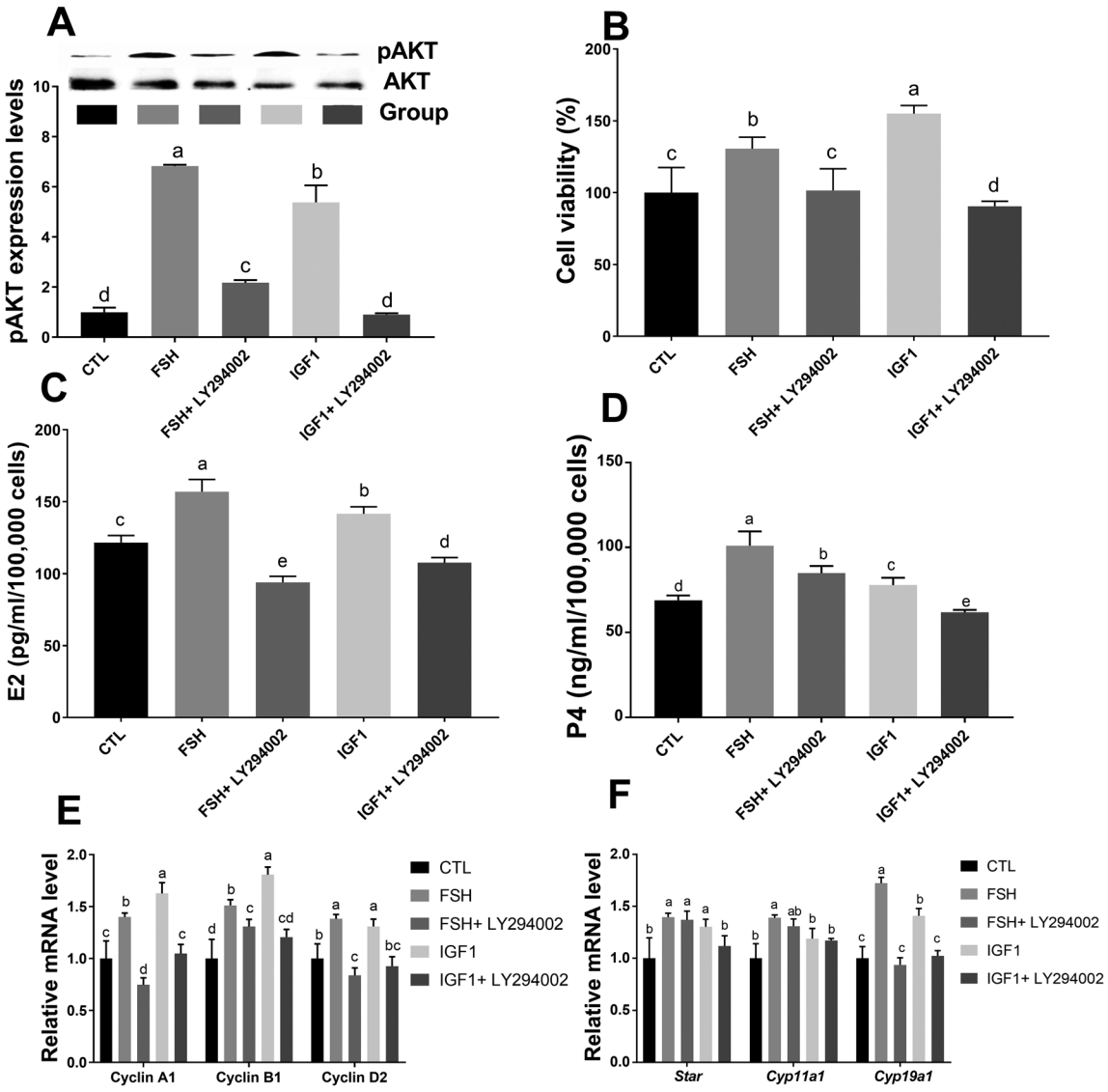


**Fig. 6.** AKT is associated with cell proliferation and hormone secretion. (A) The expression of pAKT in different groups. (B) Cell viability was measured using CCK8 assay. (C) $E_2$ levels in the culture medium. (D) $P_4$ levels in the culture medium. (E) The mRNA levels of Cyclin A1, Cyclin B1, and Cyclin D2. (F) The mRNA levels of Star, Cyp19a1, and Cyp11a1. Protein expression of pAKT was normalized to total AKT. The data are presented as mean ± SEM of three independent experiments. The letters represent significant differences ($P<0.05$).

decreased concentration of $E_2$ following IRS2 knockdown. The possible reason for $E_2$ reduction could be the decrease in the expression of *Star* (the protein associated with the transport of cholesterol across the mitochondrial membrane), *Cyp11a1* (the rate-limiting enzyme in progesterone synthesis), and *Cyp19a1* (the enzyme responsible for androgen aromatization to estrogen) [27]. Additionally, decreased $E_2$ secretion is characteristic of an atretic follicle [28]. Therefore, we inferred that IRS2 can stimulate follicular development by promoting granulosa cells proliferation and $E_2$ synthesis. These data are similar to the data from our previous study which reported that IRS2 depletion inhibits cell proliferation and decreases hormone secretion in mouse granulosa cells. However, in the present study we studied the role of IRS2 further and mainly focused on the role of miR-431 and signal transduction by targeting IRS2 in human granulosa cell.

miRNAs are involved in a wide variety of functions in the ovaries including the formation of primordial follicles, follicular recruitment and selection, follicular atresia, oocyte-cumulus cell interaction, granulosa cells function, and luteinization [29]. Moreover, several studies have reported that hormonal levels can regulate miRNA expression (including miR-431) in the ovary in the rodent species [17, 21, 30]. Our data revealed that miR-431 overexpression inhibited granulosa cell proliferation, impaired $E_2$ synthesis, and suppressed AKT activation and related gene expression, with similar functional defects in IRS2-knockdown cells. These results indicated that miR-

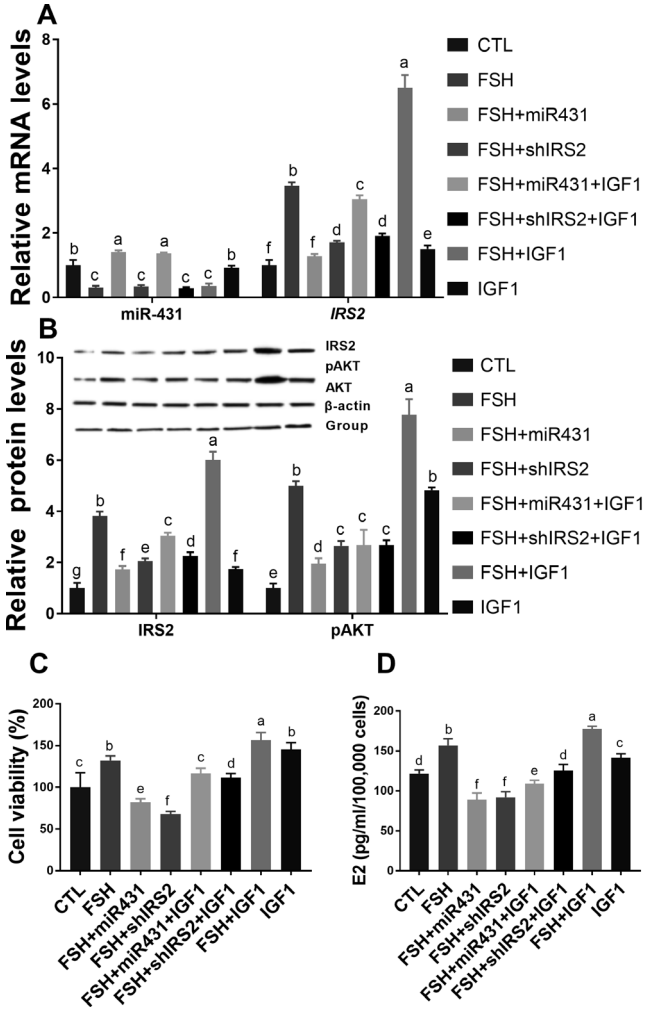


**Fig. 7.** miR-431 regulates granulosa cell function through insulin receptor substrate 2 (IRS2)/AKT. (A) The mRNA levels of miR-431 and *IRS2*. (B) Protein expression levels of pAKT and IRS2. (C) Cell viability was measured using CCK8 assay. (D) $E_2$ levels in the culture medium. Protein expression of IRS2 and pAKT were normalized to β-actin and AKT, respectively. The data are presented as mean ± SEM of three independent experiments. The letters represent significant differences ($P<0.05$).

431 may play an important role in the regulation of granulosa cell proliferation and follicular development by targeting *IRS2*.

A previous study reported that *IRS2* was a target of miR-431 [22]. Besides, IRS2-knockdown and miR-431-overexpressing cells share similar cell phenotype and the three target prediction algorithms indicated that *IRS2* was a candidate target of miR-431. Therefore, assuming that *IRS2* was a direct target of miR-431, we sought to analyze the direct relationship between miR-431 and *IRS2*. Our results showed that miR-431 overexpression inhibited the expression of IRS2, both at mRNA and protein level. This could be because miRNAs inhibit gene expression by interacting with target sites located in 3′ UTR of mRNAs, which leads to translational repression or mRNA degradation [31]. Further, we used luciferase reporter assays to test our hypothesis and the results indicated that *IRS2* was a direct target of the miR-431. Collectively, these results showed that miR-431 inhibits normal function through targeting *IRS2*.

The PI3K/AKT signaling pathway is known to promote granulosa cell proliferation [32]. However, the role of AKT in FSH-induced human granulosa cells is still not fully understood. We found that inhibition of AKT using LY294002 abrogates FSH and IGF1-mediated cell proliferation and expression of cell cycle factors. These results indicated that PI3K/AKT signaling pathway is involved in FSH-induced COV434 cells proliferation consistent with results of some previous studies using other cells [33, 34]. The PI3K/AKT signaling pathway is also associated with hormone secretion [35–37]. We observed that inhibition of the PI3K/AKT signaling pathway decreased $E_2$ production. The reduction in $E_2$ could be due to a decrease in the expression of *Cyp19a1*, which is the rate-limiting enzyme in controlling androgen aromatization to $E_2$ [26]. However, inhibition of PI3K/AKT using LY294002 showed no obvious effect on the expression of *Star* and *Cyp11a1*, unlike that of IRS2 knockdown and miR-431 overexpression groups. Although LY294002 has an inhibitory effect on PI3K, it may also target additional kinases related and unrelated to the PI3K family [38].

We further investigated the relationship between IRS2, AKT, and miR-431 in FSH-induced cell proliferation and $E_2$ synthesis. The data showed that IGF1-activated PI3K/AKT signaling pathway partly rescues miR-431 overexpression- and IRS2 knockdown-induced pAKT decrease, cell growth inhibition, and $E_2$ downregulation. These results further explained that miR-431 regulates granulosa cell function through the IRS2/PI3K/AKT signaling pathways. Moreover, the results showed that IGF1 regulated IRS2 and PI3K/AKT signaling pathways but not miR-431, suggesting that IGF1 can not only activate PI3K/AKT signaling pathway but also induce IRS2 expression to affect granulosa cell function. Although IRS2 was successfully suppressed by sh-IRS2 or miR-431 lentiviral vectors, the remaining IRS2 may activate PI3K/AKT pathway by IGF1 to regulate granulosa cell function. In addition, we also found that IGF1 promoted cell proliferation and $E_2$ synthesis together with FSH in granulosa cells as reported in previous studies in bovine [2] and mouse granulosa cells [36].

In summary, the present study demonstrated that miR-431 inhibits FSH-induced granulosa cell proliferation and $E_2$ synthesis by targeting IRS2, and this process depends on the PI3K/AKT signaling pathway (Fig. 8). A better understanding of the role of miRNAs in follicular development may provide insights into treating female infertility or designing new female nonsteroidal contraceptives.

**Conflict of interest:** The authors declare that they have no competing interests.

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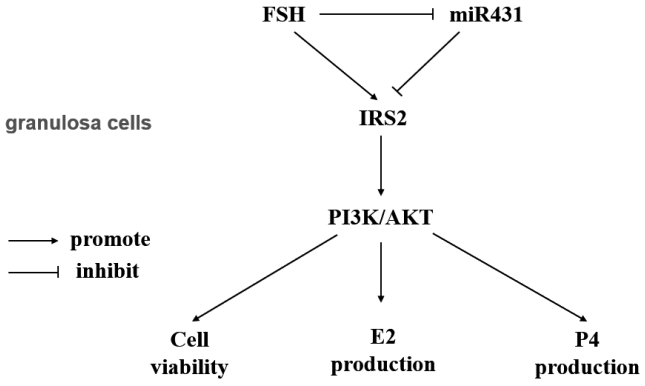


**Fig. 8.** Schematic pathway diagram summarizing the finding of the study.

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