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IRS2 depletion inhibits cell proliferation and decreases hormone secretion in mouse granulosa cells

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Running head: IRS2 is related with cell function
Abstract

Insulin receptor substrate 2 (IRS2) is a component of the insulin/insulin-like growth factor 1 (IGF1) signaling cascade, which plays an important role in mouse hypothalamic and ovarian functions. The present study was conducted to investigate the role of IRS2 in steroidogenesis, apoptosis, cell cycle and proliferation in mouse granulosa cells (GCs). Flow cytometry and CCK8 assay showed that IRS2 knockdown inhibited cell proliferation, reduced cell viability, and increased apoptosis in GCs. The study also revealed that the expression of Cyclin A1, Cyclin B1 and Bcl2 was downregulated, while the expression of Bax, Cyclin D1 and Cyclin D2 was upregulated. ELISA analysis showed that IRS2 knockdown decreased the concentrations of estradiol (E2) and progesterone (P4), which was further validated by the decreased expression of Star, Cyp11a1, and Cyp19a1. Moreover, IRS2 knockdown altered the expression of Has2 and Ptgs2, which are essential for folliculogenesis. In addition, we found that IRS2-mediated cell viability and hormone secretion are dependent on the PI3K/AKT signaling pathway. Collectively, this study demonstrated that IRS2 plays an important role in the regulation of cell proliferation and steroidogenesis in mouse GCs via the PI3K/AKT signaling pathway.

Keywords: Granulosa cells, IRS2, AKT, Cell proliferation, Steroidogenesis

Introduction

Folliculogenesis is the process of ovarian follicle development and involves follicular growth and follicular atresia. The fate of a follicle is dependent on a delicate balance of the expression and actions of factors stimulating follicular cell proliferation, differentiation and growth, and of those inducing apoptosis [1-3]. Follicular growth is facilitated by granulosa cell (GCs) proliferation, differentiation, cell cycle control, and follicular fluid formation. However, only a small fraction of the follicles undergo ovulation, while majority of them are lost before ovulation by atresia, which is a common physiological phenomenon during the overall process of follicular growth and development. This degenerative process is initiated or caused by apoptosis of GCs [4, 5].

GCs produce estradiol (E2), progesterone (P4) and insulin-like growth factor (IGF) in the ovarian micro-environment and promote the growth of delicate oocytes. Therefore, cultured GCs are essential
models for elucidating the underlying molecular mechanisms of gene regulation during folliculogenesis [1, 6]. Insulin receptor signaling has complex roles in ovarian function, including the regulation of ovarian steroidogenesis, follicular development and GC proliferation [7]. The strong association between insulin resistance and ovarian dysfunction in polycystic ovarian syndrome also suggests a role of insulin signaling in ovarian function [8, 9]. The insulin receptor substrate (IRS) proteins are the classical substrates and signaling intermediates of the insulin and IGF1 receptor. The binding of IGF1 and IRS activated phosphoinositol 3-kinases (PI3K) subsequently induces AKT activation by phosphorylation to regulate cell function [10].

The mammalian IRS protein family contains at least four members: IRS1 and IRS2, which are widely expressed; IRS3, which is mainly found in adipose tissue, and IRS4, which is expressed in the thymus, brain, and kidney [11, 12]. IRS2 has been known to mediate metabolic and reproductive functions. Previous studies have emphasized its role in growth and proliferation via PI3K/AKT signaling pathway and in the expression of cell cycle-related factors [12-14]. Withers et al., reported that IRS2 is critical for peripheral carbohydrate metabolism and β-cell function [15, 16]. Moreover, the specific role of IRS2 protein in regulating reproductive functions has been foreshadowed by knockout studies [12]. A study by Burks et al., suggested that mice lacking IRS2 have small, anovulatory ovaries with reduced number of follicles during ovarian development [17]. Female IRS2 null mice are also infertile due to reduced pituitary LH levels and gonadotroph cell numbers. Additionally, they exhibit reduced gonadotropin stimulated ovulation and markedly reduced number of ovarian follicles and corpora lutea [8, 17].

The above studies demonstrate an important role of IRS2 in female reproduction. However, the function and signaling pathway of IRS2 in mouse GCs needs to be further explored. Therefore, in this study, we aimed to investigate the role, function and signaling pathway of IRS2 in mouse GCs.

Materials and Methods

Animals and chemicals

Immature female Kunming mice (SPF grade, 21 days old) were purchased from the Laboratory Animal Central of Jiujiang University. All mice were fed a typical diet of lab chow and housed in a single room under conditions of constant temperature (~25-28 ±2°C), humidity (55 ± 5%), and...
lighting (12 h light, 12 h dark cycle) [18]. All procedures were approved by the Committee for the Ethics on Animal Care and Experiments of Jiujiang University. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. IGF1 was prepared as 8 μg/ml stock solution in distilled PBS. Stock solution of 5 mM AKT inhibitor LY294002 was prepared in DMSO and stored at -20°C. The final concentrations of IGF1 and LY294002 used for culture were ~0-80 ng/ml and 5 μM, respectively.

**Isolation and culture of mouse GCs**

The immature female mice were intraperitoneally injected with 10 IU PMSG to facilitate GC proliferation. After 44 h, primary mouse granulosa cells were isolated and cultured as described previously [19-21]. Briefly, ovaries were collected under sterile conditions and placed in DMEM/F-12, purchased from Hyclone (Logan, UT, USA). The cells were collected by needle puncture method and centrifuged at 3000 rpm for 3 min. Thereafter, the supernatant was removed and the pellets were resuspended in DMEM/F12. Finally, the cells were cultured in DMEM/F12 containing 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FBS (Corning, USA), at 37°C in 5% CO₂ and 95% O₂ for 48 h.

**Transfection of mouse GCs with shRNA-IRS2 lentiviral vector**

shRNA-IRS2 and shRNA-negative lentiviral vector were purchased from Genechem (Shanghai, China). Expression of the lentiviral vector (carrying green fluorescence protein (GFP)) was used to evaluate virus-mediated transfection efficiency, under a fluorescent microscope. Transfection of mouse GCs with shRNA-IRS2 lentiviral vector was carried out, as our previous report [22]. The mouse GCs were seeded into 6-well plates, which were cultured to ~60-70% confluence and infected by addition of 1×10⁸ TU/ml lentivirus (10 μl), 5 μg/ml polybrene and complete medium. After 10 h of incubation, the lentivirus solution was replaced by complete culture medium and cultured for 48 h. The efficiency of shRNA-IRS2 mediated knockdown was also examined by western blot and real-time quantitative PCR (RT-qPCR).

**Cell viability assay**

The cells were seeded 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 measured at 450 nm by a Microplate Reader (Bio-Rad 680). The experiments were performed in triplicate.

**RT-qPCR**
Total RNA was extracted from transfected mouse GCs using TRIzol (Sigma, USA) and stored at -80°C. Quantity and purity were checked using Nanodrop Spectrophotometer. RNA was reverse transcribed using the PrimeScript RT Reagent Kit (TaKaRa, China), following the manufacturer’s protocol. RT-qPCR was performed using the SYBR Premix Ex Taq II (Thermo Fisher Scientific Inc., USA) on the Quant Studio 6 Flex Real-time PCR system [23]. Sequences of the specific primers used to detect the related genes are listed in Supplementary Table 1. β-actin served as a reference gene. Data were quantified using the 2^ΔΔCt method, and at least three biological replicates were performed for each sample.

**Western blot**

Mouse GCs were collected and washed twice with PBS, followed by lysis with RIPA buffer (Santa Cruz, USA) containing PMSF. The total protein concentration was measured by BCA assay (Dingguo Co., Beijing, China), and 50 µg of protein, from each sample, was subjected to gel electrophoresis. Proteins were separated on 12% polyacrylamide gel before transferring them to PVDF membranes (Millipore, Bedford, MA, USA). After blocking in TBST, supplemented with 5% skim milk at 25°C for 1 h, the membranes were incubated overnight at 4°C with primary antibodies (IRS2, 1:800, Cell Signaling Technology; Cyp19a1, 1:800, Cyp11a1, 1:800, Star, 1:800, Bax, 1:800, Bcl-2, 1:800, β-actin, 1:1000, Santa Cruz). After washing, the membranes were incubated with secondary antibody, conjugated to horseradish peroxidase at 37°C for 30 min. The immunoreactive bands were visualized using a Super Signal West Pico kit, according to the manufacturer’s instructions, and the protein band densities were semi-quantified by densitometric analysis using ImageJ.

**Measurement of E2 and P4**

After 48 h of treatment, the mouse GCs were counted. Thereafter, E2 and P4 in the culture supernatant were measured by an ELISA kit (Ji Yin Mei, Co.Ltd., Wuhan, China), according to the manufacturer’s instructions. The sensitivity and inter- and intra-assay coefficients of variations of E2 and P4 ELISA kit were as follows: E2, 10 pg/ml, < 15%, and < 10%; P4, 0.25 ng/ml, < 15%, and < 10%. Each sample was measured in triplicate.

**Cell cycle analysis**

Mouse GCs, of the respective experimental groups, were collected and fixed in ice-cold 70% ethanol at 4°C for 24 h. Thereafter, the cells were washed with PBS and stained with a propidium iodide/RNase A solution for 30 min, at 25°C, in the dark. Finally, the cells were analyzed by flow
cytometry using a BD FACSCalibur system and ModFit LT for MacV3.0 software. A minimum of 20,000 cells were analyzed. All experiments were repeated three times.

Cell apoptosis analysis

Mouse GCs of the respective experimental groups were collected and quantified using an Annexin V-PE and 7-AAD kit, after culturing for 48 h. The GCs were harvested, washed with PBS, and digested with 0.25% trypsin, without EDTA. Thereafter, the cells were centrifuged at 500×g for 5 min, washed twice with cold PBS, and their density adjusted to 1×10⁵ cells per milliliter. The cells were then treated following the manufacturer’s instructions. First, cells were resuspended in 50 µl binding buffer. Secondly, 5 µl of 7-AAD was added and the mixture was allowed to stand for 15 min in the dark. Finally, 450 µl binding buffer and 1 µl Annexin V-PE were added and incubated for 15 min in the dark. Detection by flow cytometry was performed within 1 h. The experiment was repeated independently three times.

Statistical analysis

All experimental data were analyzed using one-way ANOVA. Analysis was done using SPSS software (Version 13.0; SPSS, Inc., Chicago, IL, USA) with Tukey’s post hoc test. P < 0.05 was regarded as statistically significant. All data are represented as mean ± SEM, from at least three separate experiments.

Results

IRS2 knockdown inhibits mouse GC proliferation

Flow cytometry analysis showed that the percentage of IRS2 knockdown GCs in S phase was higher than that of the shRNA-negative group (Fig. 1A). However, the ratio of cells in G1 phase significantly decreased, and there was no significant difference in G2 phase (Fig. 1A). The CCK8 results indicated that IRS2 knockdown decreased the percentage of cell viability in IRS2 knockdown groups compared to control groups (Fig. 1B). The mRNA levels of cell cycle factors (Cyclin A1, Cyclin B1, Cyclin D1 and Cyclin D2) indicated that IRS2 knockdown significantly decreased the mRNA expression of Cyclin A1 and Cyclin B1, but increased the expression of Cyclin D1 and Cyclin D2 (Fig. 1C- F).

IRS2 knockdown decreases hormone secretion in mouse GC culture medium
ELISA results showed that the levels of E\textsubscript{2} and P\textsubscript{4} were lower in the shIRS2 group than in the shRNA-negative group (Fig. 2A and Fig. 2B). Furthermore, the mRNA or protein expression levels of IRS2 was significantly decreased, suggesting that shRNA-IRS2 lentivirus efficiently inhibited IRS2 expression (Fig. 2C). The mRNA or protein expression levels of Star (the protein associated with the transport of cholesterol across the mitochondrial membrane) and Cyp11a1 (the rate-limiting enzyme in P\textsubscript{4} synthesis) were both significantly reduced, while the expression levels of Cyp19a1 (the enzyme responsible for androgen aromatization to E\textsubscript{2}) was slightly decreased, but showed no statistically significant difference with shRNA-negative group (Fig. 2C-E).

**IRS2 knockdown promotes mouse GC apoptosis**

The results showed that the apoptosis rate of the shRNA-IRS2 group was significantly higher than that of the shRNA-negative group (Fig. 3A). Furthermore, we measured the protein and mRNA levels of apoptotic regulatory genes, Bcl2 and Bax, to further elucidate the effects of IRS2 knockdown on mouse GC apoptosis. The results indicated that the expression of Bcl2 was significantly decreased, and the expression of Bax was increased at the protein level, but no difference was observed at the mRNA expression level, between the shRNA-IRS2 and shRNA-negative groups (Fig. 3B-E).

**Effects of IGF1 on activation of AKT and IRS2 expression**

It has been previously reported that AKT is a downstream regulator of IRS2 [24]. To further validate the relation of IRS2 and AKT in mouse GCs, IRS2 was induced by different doses of IGF1 and cultured for different time intervals. The results showed that pAKT was increased at 15 min, peaked at ~30-60 min and was still high at 24 h, but began to decrease within 48 h after culturing with 40 ng/ml IGF1 (Fig. 4A). The IRS2 mRNA was increased at 12 h and peaked at 24 h (Fig. 4B). We also treated the mouse GCs with different doses of IGF1 (0, 10, 20, 40 and 80 ng/ml) for 24 h. The results showed that IGF1 induces the expression of IRS2 and the pAKT in a dose-dependent manner. Moreover, 20 ng/ml IGF1 was enough to active AKT, but the lowest concentration of IGF1 to induce IRS2 expression was 40 ng/ml (Fig. 4C and D).

**IRS2/AKT is necessary for IGF1-induced hormone secretion**

To determine the role of IRS2/AKT signaling pathway in IGF1-induced hormone secretion, we measured the concentrations of E\textsubscript{2} and P\textsubscript{4} in the mouse GC culture medium. We found that 40 ng/ml IGF1 promoted E\textsubscript{2} and P\textsubscript{4} secretion (Fig. 5A and B) and increased the mRNA and protein levels of Star, Cyp19a1, and Cyp11a1 (Fig. 5C and D). In addition, IRS2 silencing or 5 μM LY294002, clearly
inhibited the IGF1-stimulated hormone secretion (Fig. 5A and B) and the expression of Star, Cyp19a1, and Cyp11a1 (Fig. 5C and D).

**IRS2/AKT is necessary for IGF1-induced cell viability**

The CCK8 assay indicated that the rate of cell viability was higher in the shRNA-negative or DMSO group after treatment with IGF1, compared with the other groups (Fig. 6A). Moreover, IGF1 induced-pAKT was significantly impaired by IRS2 silencing or LY294002 (Fig. 6C). At the same time, IRS2 silencing or LY294002 altered the expression of the apoptotic regulatory genes, Bcl2 and Bax. The expression of Bcl2 was downregulated, while the expression of Bax was upregulated compared to the related control group (Fig. 6B and C). In addition, inhibition of AKT by LY294002 had no obvious effect on IGF1-induced the expression of IRS2 (Fig. 6B and C).

**IRS2/AKT is involved in the expression of key genes associated with folliculogenesis**

We also studied the expression of key genes (Has2 and Ptgs2) associated with folliculogenesis, ovulation, and luteinization in mouse GCs. The results showed that inhibition of IRS2/AKT signaling by RNAi or LY294002, significantly decreased the mRNA expression level of Has2 and Ptgs2 (Fig. 7A and B), suggesting that IRS2/AKT may be involved in the process of folliculogenesis, ovulation, and luteinization, in the mouse ovary.

**Discussion**

Previous studies have demonstrated that IRS2 is a key regulatory factor in metabolic and reproductive functions in vivo [15,17, 25]. However, little information is available on the role of IRS2 in cell cycle control, proliferation, apoptosis, and steroid hormone synthesis at cellular levels in GCs. To address these questions, we determined the function of IRS2 in mouse GCs, in this study.

Previous studies have reported that IRS2 expression was found to increase with follicular growth, [8] and FSH and IGF1 induced IRS2 expression in GCs [12], indicating that IRS2 may play an important role in follicular development. In our study, we found that IRS2 knockdown inhibits mouse GC proliferation in vitro; this result supports the previous in vivo study, where IRS2 null female mice exhibited decreased primary oocytes, follicles, and corpora lutea, compared to wild-type controls [17], suggesting that IRS2 plays a role in the growth and development of oocytes and follicles. A plausible explanation is that IRS2 activates the PI3K/AKT pathway, which has been earlier identified as a crucial mediator of oocyte growth, granulosa cell proliferation and follicular development [26, 27].
GCs play a crucial role in female reproduction by synthesizing sex hormones, including E\(_2\) and P\(_4\), as well as interacting with the oocytes [28, 29]. Therefore, we speculate that IRS2 may participate in steroid hormone synthesis to regulate follicular growth and ovarian steroidogeneses. Thus, in this study, we demonstrated that the concentrations of E\(_2\) and P\(_4\) were significantly decreased in the IRS2 silenced group compared to the shRNA-negative group. In the process of progesterone synthesis, transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane by Star, and conversion of cholesterol into pregnenolone by Cyp11a1, are the rate limiting steps, which take place in the mitochondria [30, 31]. In the present study, we found that IRS2 silencing leads to decreased expression of Star and Cyp11a1. This result confirms that IRS2 may be involved in the growth and development of oocytes and follicles. Decrease in the levels of the E\(_2\) and P\(_4\) could be attributed to the decrease in Star and Cyp11a1 expression.

In view of the importance of cell cycle regulation in follicular development, we evaluated the mouse GC cell cycle, after IRS2 knockdown. Our results indicated an increase in the S phase compared to the shRNA-negative group, accompanied by a decrease in the G1 phase. IRS2 knockdown decreased the mRNA level of Cyclin A1 and Cyclin B1, but not Cyclin D1 and Cyclin D2. Cyclin A1, being a key regulator of cell cycle progression from the S phase to the G2/M phase, reduction in Cyclin A1, resulted in the S phase arrest. Furthermore, Cyclin B1 may function as a key regulator of cell cycle progression from S phase to G2/M phase, and is associated with cyclin-dependent kinase1 (CDK1) [6, 32]. Thus, we surmised that reduced mRNA expression of Cyclin B1 and Cyclin A1 may inhibit cell cycle progression from S to G2/M phase, after IRS2 knockdown. Moreover, the mRNA levels of Cyclin D1 and Cyclin D2 were up-regulated after IRS2 knockdown. This may be attributed to the fact that Cyclin D1 represses DNA replication by binding to proliferating cell nuclear antigen (PCNA) and Cdk2 [5, 33, 34] and Cyclin D2 promotes the G1 to S phase transition in a coordinated fashion, together with its antagonist p27Kip1 [35].

In female mammals, it is estimated that there is a storage capacity for tens of thousands of primordial follicles at birth, but only a small fraction of these are selected for ovulation. More than 99% of follicles undergo atresia, which is a common physiological phenomenon during the overall process of follicular growth and development [4, 36]. Previous studies have suggested that GC apoptosis is the main cause of follicular atresia [37, 38], and is regulated by Bax and Bcl2 gene family members [6, 39]. IRS2 may have evolved to acquire a unique role in this regulation. Thus, we hypothesized that
IRS2 may promote cell survival by attenuating mouse GC apoptosis. Our results showed that IRS2 knockdown has significant effect on apoptosis in mouse GCs, as determined via flow cytometry. At the transcriptional level, Bcl2 was significantly decreased by IRS2 knockdown at mRNA and protein levels. However, the mRNA level of Bax showed no significant effect on apoptosis of mouse GCs.

Previous studies have reported that GCs express IGF1 during the early follicular phase and high levels of IGF1 were found in healthy follicles [40]. Some studies have reported that IGF1 affects GC proliferation, aromatase activity and P4 synthesis [41, 42]. Moreover, IGF1 is capable of augmenting FSH-mediated E2 synthesis [43, 44]. In addition, IGF1 binding to its receptors on the cell surface activates IRS2 signaling, which further activates the PI3K/AKT pathway to influence cell function [45-48]. The potential relation between IRS2, AKT, cell viability and hormone secretion have been examined in mouse GCs. The results showed that IGF1 stimulates the expression of IRS2 and activates AKT in mouse GCs, which is consistent with previous reports [47-49]. Moreover, IRS2 knockdown inhibited IGF1-stimulated pAKT, cell viability and hormone secretion. Further, studies have revealed that IGF1-induced pAKT, cell viability and hormone secretion can be attenuated by LY294002. This finding is similar to the results of a previous study that showed that the IGF1 induces AKT phosphorylation and cell proliferation in human GCs [47] and bovine GCs [43].

In addition, we also investigated IRS2/AKT signaling in folliculogenesis; we detected the key genes as Has2 (the key enzyme in the production of hyaluronic acid) and Ptgs2 (the key enzyme in prostaglandin biosynthesis, mainly expressed in GCs in the ovaries) [30, 50] to be associated with folliculogenesis and ovulation. We found that transcription of Has2 and Ptgs2 was down-regulated after IRS2 knockdown. The decrease of Has2 and Ptgs2 are an indication of hyaluronan shortage during cumulus expansion, and affects ovulation and corpus luteum formation. Accordingly, we speculated that IRS2 may participate in folliculogenesis, ovulation, and luteinization by regulating the expression of these genes in the mouse ovary.

In summary, this study provides additional evidence that IRS2 plays an important role in folliculogenesis, granulosa cell proliferation, ovulation, hormone secretion and luteal tissue formation. Previous studies have revealed that this process is related to the PI3K/AKT signaling pathway. The results of our study adds to the existing knowledge on the role of IRS2 and will help to better understand the function of IRS2 in the female reproductive process.
Acknowledgements

None.

Conflict of interest statement

The authors declare that they have no competing interests.

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Fig. 1. IRS2 knockdown inhibits mouse GC proliferation. (A) Cell cycle analysis was performed by flow cytometry. (B) Cell viability was measured by CCK8 assay. (C-F) The mRNA levels of cell cycle factors (Cyclin A1, Cyclin B1, Cyclin D1 and Cyclin D2). The data are presented as mean±SEM of three independent experiments. Bars with different letters are significantly different (P<0.05).
Fig. 2. IRS2 knockdown decreases hormone secretion in mouse GC culture medium. (A-B) E$_2$ and P$_4$ levels in mouse GC culture medium. (C-E) The mRNA and protein levels of IRS2, Star, Cyp19a1 and Cyp11a1. Protein expression was normalized to β-actin. The data are presented as mean±SEM of three independent experiments. Bars with different letters and asterisks are significantly different (P<0.05).
**Fig. 3.** Effect of IRS2 knockdown on cell apoptosis. (A) Cell apoptosis was detected by flow cytometry. (B-E) The mRNA and protein expression levels of Bcl2 and Bax. A1: Necrotic cells; A2: Progressed apoptotic cells; A3: Viable cells; A4: Early apoptotic cells. Protein expression was normalized to β-actin. The data are presented as the mean±SEM of three independent experiments. Bars with different letters are significantly different (P<0.05).
Fig. 4. Effect of IGF1 on activation of AKT and expression of IRS2 in mouse GCs. (A) The expression of AKT and pAKT after adding IGF1 at different time intervals. (B) The mRNA expression of IRS2 after adding IGF1 at different time intervals. (C) The expression of AKT and pAKT after adding different doses of IGF1 at 24 h. (D) The mRNA expression of IRS2 after adding IGF1 at 24 h. The pAKT was normalized to total AKT. The data are presented as the mean±SEM of three independent experiments. Bars with different letters are significantly different (P<0.05).
**Fig. 5.** IRS2/AKT signaling is necessary for IGF1-induced hormone secretion. (A-B) Concentrations of E$_2$ and P$_4$ in mouse GC culture medium at 48 h. (B-C) The mRNA and protein expression levels of Star, Cyp19a1 and Cyp11a1 at 24 h. Protein expression was normalized to β-actin. The data are presented as the mean±SEM of three independent experiments. Bars with different letters are significantly different (P<0.05).
Fig. 6. IRS2/AKT signaling is necessary for IGF1-induced cell viability. (A) Cell viability was measured by CCK8 at 24 h. (B-C) The mRNA and protein levels of IRS2, Bcl2 and Bax and the protein expression level of pAKT/AKT at 24 h. Protein expression was normalized to β-actin. The data are presented as the mean±SEM of three independent experiments. Bars with different letters are significantly different (P<0.05).
Fig. 7. Effects of IRS2/AKT signaling by RNAi or LY294002 on the expression level of the key genes associated with folliculogenesis in mouse GCs. (A-B) The mRNA expression of Has2 and Ptgs2 in different groups. The data are presented as the mean±SEM of three independent experiments. Bars with different letters are significantly different (P<0.05).
## Supplementary Table 1 Primer sequences used for RT-qPCR

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<th>Gene</th>
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<th>Reverse primer sequences (5’→3’)</th>
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