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**—Original Article—**

# Immunolocalization and Expression Pattern of Gpr3 in the Ovary and Its Effect on Proliferation of Ovarian Granulosa Cells in Pigs

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**Abstract:** Gpr3, a member of the G protein-coupled receptor superfamily, was known as a critical factor for the maintenance of meiotic prophase arrest in oocytes via a Gs protein-mediated pathway. The present studies were conducted to examine the ovarian immunolocalization of Gpr3, its expression pattern in different stages of fetal, postnatal and developmental pigs and its effect on proliferation of ovarian granulosa cells in pigs. Immunohistochemical analysis indicated that Gpr3 was localized in egg nests, oocytes and granulosa cells (GCs) of the follicle ranging from the primordial to Graafian stages and the corpora lutea. Staining was faintly present in the corpora lutea and weak in GCs but was strong in oocytes. Real-time PCR and Western blotting indicated that Gpr3 mRNA and protein were both present in the different ages of ovaries, and there were wavy changes in the expression levels from postpartum 1 to 180 days. Moreover, both the mRNA and protein levels of Gpr3 were upregulated significantly during follicle growth, suggesting that Gpr3 might play potential roles in regulating ovarian follicle development in the pig. MTT and flow cytometry analyses indicated that Gpr3 knockdown significantly promoted proliferation of porcine GCs while increasing the proportion of cells in the S phase and the expression of Cyclin B1 and Cyclin D2, providing new insights into how Gpr3 signaling regulates the proliferation of porcine GCs. In conclusion, the stage- and cell-specific expression pattern of Gpr3 in the porcine ovary suggested that Gpr3 might play an important role during the entire process of follicular development and luteinization.

**Key words:** Expression pattern, G protein-coupled receptor 3 (Gpr3), Granulosa cells, Ovary, Pig

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The mammalian ovary is a heterogeneous organ [1], composed primarily of follicles and corpora lutea (CL) in various stages of developmental progression [2]. Folliculogenesis is a dynamic process that begins during fetal development in pigs. In the porcine embryo, primordial germ cells are first detectable at 18 days post coitum (dpc), and proliferation of oogonia starts during their migration to the genital ridge. Oogonia continue to divide and reach the peak of mitotic activity at 50 dpc [3]. Oocytes are first found at approximately 40 dpc [4] and arrest at the diplotene stage of the first meiotic prophase. The oogonia and oocytes are present within germ cell clusters, also referred as egg nests [5]. As a subset of oocytes within individual dying egg nests, the syncytia undergoes programmed breakdown [6]. This process ends with the formation of primordial follicles [7]. The oocyte enclosed within primordial follicles remains arrested in the first meiotic prophase until a preovulatory surge of luteinizing hormone (LH) acts on the follicle cells surrounding the oocyte to stimulate meiotic resumption, as well as ovulation [8]. After ovulation, GCs and theca cells begin to differentiate into luteal cells, and then the corpora lutea form. The orderly running of the whole process is attributed to the regulation and coordination by a variety of signaling pathways, such as G protein-coupled receptor signals.

G protein-coupled receptors (GPCRs), as the largest family of cell-surface receptors, respond to a diverse array of ligands and mediate cellular responses to hormones, peptides, lipids and neurotransmitters, as well as the senses of smell and taste [9]. Gpr3 is one of the orphan GPCRs members, although some effects of sphingolipids have been reported [10]. The *Gpr3* gene was first isolated from a mouse brain cDNA library [11] and is strongly expressed in the ovary, testis and cerebral tissues [11–13]. Recently, the Gpr3 receptor was characterized in several cultured cells as a constitutive activator of adenylyl cyclase in the absence of any added agonist [12] and was shown to play a major role in maintaining meiotic prophase arrest of the mouse oocyte via a Gs protein-mediated pathway [14–16]. In addition, inability to sustain concentrations of Cyclic AMP (cAMP) in oocytes from mice deficient in Gpr3 led to precocious gonadotropin-independent meiotic resumption, which interrupted the synchrony between oocyte maturation and ovulation, leading to premature ovarian aging and loss of fertility [17]. These findings indicated that the Gpr3 signaling pathway was one of the most critical pathways for regulation of the oocyte maturation and follicular development.

However, to date, the localization and expression pattern of Gpr3 during follicular development in the mammalian ovary has not been well documented. In particular, its effect on proliferation of ovarian GCs has not been mentioned yet. To enhance our understanding of the porcine Gpr3 receptor, the temporal and spatial localization of Gpr3 protein in the porcine ovary during fetal and postnatal development stages was determined by immunohistochemistry, the mRNA and protein expression levels in the ovary including follicles were measured by real-time RT-PCR and Western blotting, and the

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cell proliferation of ovarian GCs was detected by MTT and flow cytometry.

## Materials and Methods

*Animals and ovarian sample collection*

Three-way cross pigs (Duroc, paternal; Large White;Landrace, maternal) were obtained from the Changxing Ecological Agriculture (Wuxi, China). The animals were kept indoors and provided with commercial foods three times per day and tap water *ad libitum*. Ovaries were immediately collected from the crossbred pigs at 50, 70 and 90 days of gestation and at 1, 25, 35, 70, 140, 180 days old after necropsy, respectively. One ovary was fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for immunohistochemistry, and the other ovary was immediately put into liquid nitrogen for real-time RT-PCR and Western blotting. Three crossbred pigs of each age were used for ovarian sampling. The gestation day was estimated from the first mating day. The age for ovary collection was chosen to provide samples with progressively mature populations of oocytes and follicles [18]. Animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

*Immunohistochemistry*

Ovaries were fixed overnight at 4 C in 4% paraformaldehyde solution, transferred through a graded series of ethanol and xylene and then embedded in paraffin wax. Sections (thickness, 5 μm) cut on a standard microtome were mounted on 3-aminopropyl-triethoxysilane (APES)-coated microscope slides for immunohistochemical localization of Gpr3 protein as in the previous reports [19]. Briefly, sections were deparaffinized in xylene and rehydrated through a graded series of ethanol before being washed with double-distilled water. To increase epitope exposure, sections were heated for 15 min in sodium citrate buffer (0.01 M, pH 6.0) in a microwave oven. The sections were cooled and washed with 0.01 M PBS (pH 7.2), incubated in 3% $H_2O_2$ (v/v) in methanol at 32 C for 30 min to quench endogenous peroxidases, blocked with 5% Bovine Serum Albumin (BSA) in PBS for 1 h and then incubated with mouse monoclonal antibody against Gpr3 (1:100 dilution, Abcam, Shanghai, China, lot: ab55136) overnight at room temperature in a humidified chamber. The specific protein immunoreactivity was visualized with an SABC Kit (Boshide, Wuhan, China, lot: SA2001) and 0.05% (w/v) 3, 3'-diaminobenzidine tetrachloride (Sigma, St. Louis, MO, USA) in 10 mM PBS-buffered saline containing 0.01% (v/v) $H_2O_2$ for 2–3 min. Specificity of the antibody was examined using normal goat serum (NGS) instead of primary antibody. In order to identify structural components and cell morphology, the sections were counterstained with hematoxylin and mounted with coverslips. As a positive control, sections of adult porcine brain were included according to the above staining procedure. Immunostaining was evaluated with digitalized images captured with an Olympus camera. The photographic images were processed in the Photoshop software (Adobe Systems, San Jose, CA, USA).

Intensity of immunoreactive staining was scored by three independent observers using the following ratings: +, positive; +/–, weakly positive; and –, negative. Stages of follicular development were classified as follows: primordial follicles contained an oocyte surrounded by a single layer of squamous GCs; primary follicles contained an oocyte surrounded by a single layer of cuboidal GCs; secondary follicles contained an oocyte surrounded by more than one layer of cuboidal GCs but lacking an antrum; tertiary follicles contained an oocyte surrounded by multiple layers of GCs in which an antrum was apparent; and mature or Graafian follicles exhibited a large antrum and cumulus oophorus that projected into the antrum.

*Follicle collection and culture of granulosa cells*

Follicles and GCs were collected from ovaries of prepubertal commercial gilts at a local slaughterhouse. All the ovaries were kept in a thermo container filled with prewarmed physiological saline containing gentamicin (500 IU/ml) at 37 C and transported to the laboratory within 1 h. Follicles were dissected from the ovary and grouped on the basis of their diameter in small (< 3 mm), medium (3–5 mm) and large (> 5 mm) groups in accordance with a previous report [20]. Ten to thirty independent follicles in each group were used for total RNA or protein extraction.

GCs were aspirated from medium-sized follicles of 3–6 mm in diameter using a 10-ml disposable syringe connected to an 18-gauge needle and filtered through a stainless steel filter (100 μm) to remove the cumulus-oocytes complexes (COCs). The cells were centrifuged at 1000 rpm for 5 min and washed twice in Dulbecco's modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12; Gibco, Shanghai, China). Then, GCs were cultured as in our previous reports [21]. Briefly, after counting in a hemocytometer, GCs were cultured in six-well culture plates (Costar; Corning, Corning, NY, USA) at a density of $(3–5)\times10^6$ cells per well in 2 ml of DMEM/F12 medium supplemented with 10% (v/v) fetal calf serum (FCS; Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 C in a humidified atmosphere of 5% $CO_2$ in air for 24 h, and then the wells were washed with PBS to remove unattached cells. Exponentially growing cells were used for the experiments.

*Design and transfection of siRNA*

The complementary DNA (cDNA) sequence of the porcine *Gpr3* gene, recently cloned by our group (GenBank accession no. HQ606483)[22], was examined using Invitrogen's web-based siRNA design software (https://rnaidesigner.invitrogen.com/rnaiexpress/) to select appropriate siRNA target sites. A pair of oligonucleotides corresponding to *Gpr3* cDNA at the start position of 901 bp was designed. The siRNA sequence of *Gpr3* is as follows: sense, AGC AGG GUG AGA UAA GUG UAG AGG G; antisense, CCC UCU ACA CUU AUC UCA CCC UGC U. A BLAST search of these sequences confirmed their specificity to Gpr3 only. In addition, a nonsense sequence with no similar match to any known sequence was used as control. The siRNA sequence of the nonsense sequence is as follows: sense, UUC UCC GAA CGU GUC ACG UTT; antisense, ACG UGA CAC GUU CGG AGA ATT. The Gpr3 small RNA interference (RNAi) was called Gpr3-siRNA, and the nonsense sequence for Gpr3 was called negative control siRNA (NC-siRNA).

Cultures of GCs were transfected with Gpr3-siRNA or NC-siRNA using Lipofectamine™ 2000 Reagent (Invitrogen, Shanghai, China), as recommended by the manufacturer. Briefly, RNAi duplex, Lipofectamine™ 2000 and 250 μl DMEM/F12 medium were combined and incubated for 20 min at room temperature. The transfection

complex was added to the cells and incubated for 48 h at 37 C.

*RNA extraction and QRT-PCR*

Total RNA was isolated from frozen ovaries, follicles and GCs using TRIzol reagent (Invitrogen, Carlsbad, CA, US) and treated with DNase I (RNase-free) (TaKaRa, Dalian, China) to remove genomic DNA following the manufacturer's instructions. The RNA concentration and purity were determined by a standard spectrophotometer (SMOIF, Shanghai, China). A sample from the same group was mixed into an RNA pool. Five micrograms of total RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase (Fermentas, Shanghai, China) and oligonucleotide primers.

Target genes and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were quantified by real-time PCR with an ABI 7300 using a commercial kit (FastStart Universal SYBR Green Master; Roche, Nanjing, China). The gene-specific primers were designed on the basis of porcine mRNA sequences using Beacon Designer 7.0 (Table 2). The cDNA generated was used as a template for PCR. The reactions were repeated thrice for each sample in 20 µl with 200 µmol/l primers and 1 µl cDNA template using 10 µl FastStart Universal SYBR Green Master (ROX). PCR amplification was performed and consisted of 42 cycles of denaturation at 95 C for 15 sec, annealing at 58 C for 30 sec, and extension at 72 C for 30 sec, after the initial denaturation step (95 C for 3 min). The specificity of the PCR product was verified with a melting curve and by agarose gel electrophoresis. No bands were seen in mock reactions in which reverse transcriptase was omitted.

*SDS-PAGE and Western blotting*

Protein was extracted from samples in a Dounce homogenizer with RIPA lysis buffer (Beyotime, Nantong, China) for Western blot analysis. After homogenization, samples were incubated for 30 min on ice, and centrifuged at 13,000 rpm for 10 min at 4 C. The supernatant was separated, and the protein concentration was determined using a BCA Protein Assay kit (Beyotime). In each experiment, equal amounts of sample lysate (15–40 µg) were separated by 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under standard reducing conditions with a precision protein molecular weight marker (Fermentas) and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% BSA in TBST buffer (20 mM Tris-buffered saline, 0.05% Tween 20, pH 7.5) for 1 h at room temperature and incubated overnight at 4 C with diluted antibodies specific to Gpr3 (1:500; Abcam) and β-actin (1:3,000; Santa Cruz Biotechnology, Shanghai, China). Subsequently, the membrane was washed with TBST buffer and incubated with horseradish peroxidase-linked secondary goat anti-mouse IgG antibody (Santa Cruz) diluted at 1:4,000 for 2 h. Finally, the blot was washed three times and visualized with SuperSignal® West Pico Chemiluminescent Substrate kits (Pierce Biotechnology, Shanghai, China).

*Cell proliferation assay*

GCs were counted in a hemocytometer, and the viability was determined by trypan blue dye exclusion; the GCs were then seeded in 96-well culture plates (Costar; Corning) at a density of (3–5)×$10^4$ cells per well. GCs were treated with or without siRNA. The cell proliferation at the end of the process of culture was determined using an MTT kit (KeyGEN, Nanjing, China) according to the manufacturer's instructions. The optical density at 550 nm (OD550) was determined using an ELISA reader (Bio-TEK Instruments, Winooski, VT, USA).

*Flow cytometry*

In order to check the cell cycle, we seeded GCs in a 25 $cm^2$ culture flask at a density of (5–8) ×$10^5$ cells per flask. After treatment, GCs were digested using 0.25% pancreatic enzyme, washed twice with PBS, fixed in precooled 70% (v/v) ethanol overnight, and stained with 100 µg/ml propidium iodide (PI) at 4 C for 1 h. DNA content was monitored using a flow cytometer (FACScan; Becton, Dickinson, Franklin Lakes, NJ, USA).

*Statistics analysis*

Relative mRNA expressions were analyzed by using the $2^{-\Delta\Delta CT}$ method [23]. The intensity of Western blot signal was determined by digital image analysis using the NIH ImageJ software. All data were expressed as the mean ± SD. Statistical differences were calculated by one-way ANOVA and Duncan's post hoc tests using SPSS version 18.0 (SPSS, Chicago, IL, USA). The correlation between the expression levels of *Gpr3* and age of the pigs was examined by bivariate analysis, followed by Pearson correlation coefficient and a two-tail test of significance. A P value less than 0.05 was considered statistically significant. Each experiment was repeated at least three times.

## Results

*Histological observation and immunolocalization of Gpr3 protein in the ovaries of fetal and postnatal pigs*

In order to investigate the ovarian histological morphology and immunolocalization of Gpr3 protein in ovaries during follicular development, an immunohistochemical staining of Gpr3 was performed in the ovaries of different stages of fetal, postnatal and developmental pigs. Representative images of porcine ovaries at each developmental stage are shown in Fig. 1 and are discussed in more detail below. The cell-specific localization of Gpr3 protein is summarized in Table 1.

Ovaries collected on 50 dpc were comprised predominantly of stromal cells and egg nests filled with oogonia and oocytes. The egg nests surrounded by the stromal cells were located in the ovarian cortical region adjacent to the single layer surface epithelium and extended into the medulla. There was no evidence of follicle formation at this stage of development. The various cell types in the ovary could be distinguished by nuclear morphology; oogonia and oocytes had large round nuclei, whereas stromal cells contained elongated nuclei [24]. In addition, several apoptotic oogonia with severely marginated chromatin were present in the egg nests. The Gpr3 protein reactivity was limited to egg nests within the ovary. Stromal cells surrounding the egg nests did not show signs of Gpr3 protein (Table 1 and Fig. 1A). As seen at 50 dpc, ovaries recovered at 70 dpc still contained large numbers of egg nests wrapping the oogonia and oocytes, but the nests situated at the cortical-medullary

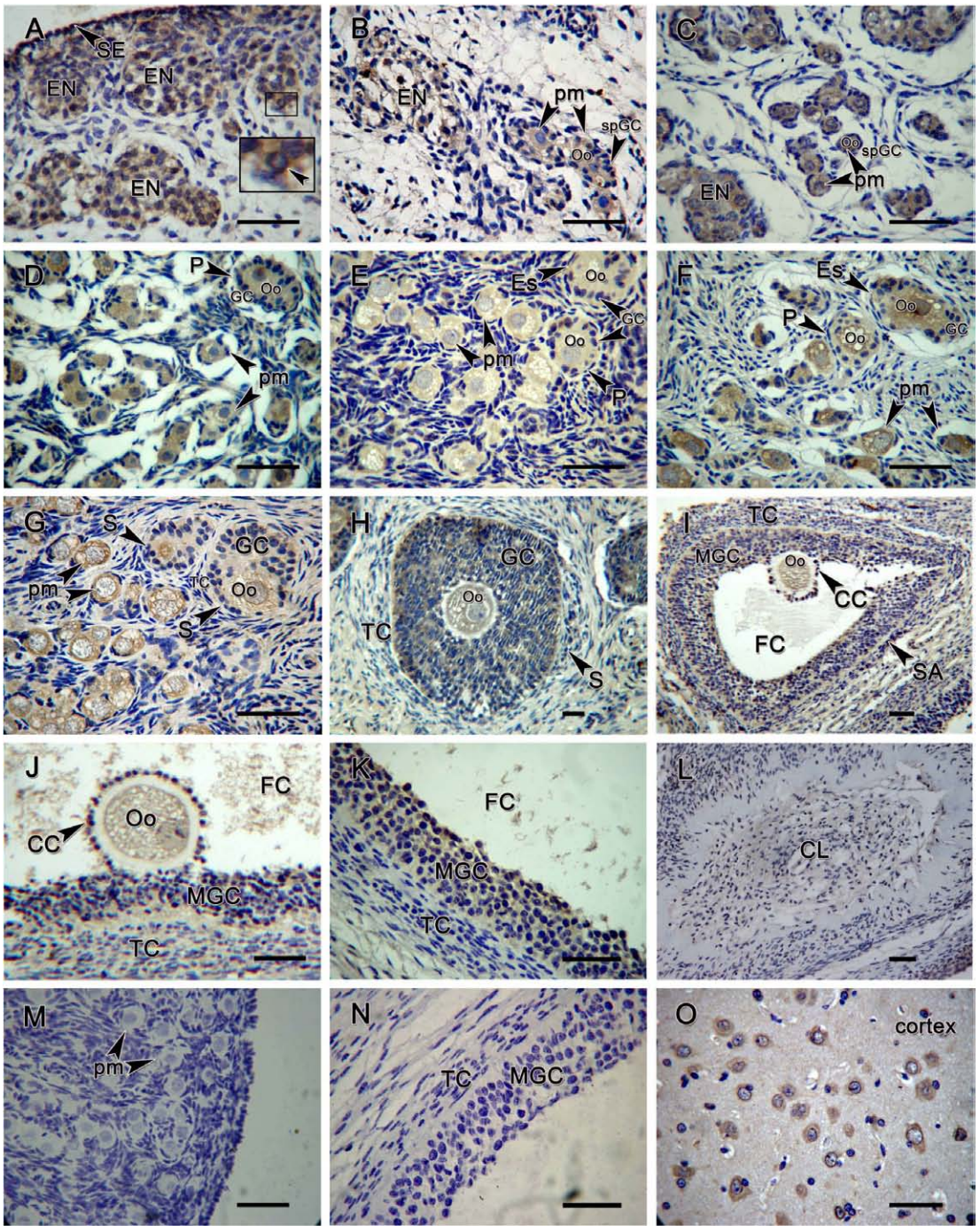


**Fig. 1.** Histoarchitecture and immunolocalization of Gpr3 proteins in the porcine ovary. Ovarian sections were immunostained for Gpr3 and counterstained with hematoxylin. The immunohistochemistry signals appear brown in color, and the counterstaining background appears blue in color. Note that Gpr3 immunoreactivity was detected in germ cells of egg nests (A) and oocyte cytoplasms; granulosa cells of primordial (C), primary (E), secondary (H), tertiary follicles (I) and Graafian follicles (J, K); and the corpus luteum (L). In control sections (M–O), no significant immunoreactivity was observed when normal goat serum was used instead of the primary antibody (M, N), and a strong signal was detected in the section of porcine cerebral cortex incubated with Gpr3 antibody (O). The ages of the ovaries, respectively, are 50 dpc (A), 70 dpc (B), 90 dpc (C), 1 dpp (D), 25 dpp (E, M), 35 dpp (F), 70 dpp (G), 140 dpp (H–I) and 180 dpp (J–L, N and O). (A): An apoptotic oogonia with severely marginated chromatin is shown (arrowhead). SE, superficial epithelium; EN, egg nest; pm, primordial follicle; P, primary follicle; Es, early secondary follicles; S, secondary follicle; SA, small antral follicle; CL, corpus luteum; Oo, oocyte; spGC, squamous pregranulosa cell; GC, granulosa cell; MGC, mural granulosa cell; CC, cumulus cell; TC, theca cell; FC, follicular cavity. Scale bars= 50 μm. Experiments were repeated at least 4 times, and representative results are shown.

**Table 1.** Summary of histologic and immunohistochemical analysis of Gpr3

| Follicular development | Staining intensity | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 50 dpc | 70 dpc | 90 dpc | 1 dpp | 25 dpp | 35 dpp | 70 dpp | 140 dpp | 180 dpp |
| **Oocyte** | | | | | | | | | |
| Egg nest | + | + | + | + | NA | NA | NA | NA | NA |
| Primordial | NA | + | + | + | + | + | + | + | + |
| Primary | NA | NA | NA | + | + | + | + | + | + |
| Secondary | NA | NA | NA | NA | + | + | + | + | + |
| Tertiary | NA | NA | NA | NA | NA | NA | NA | + | + |
| **Granulosa cell** | | | | | | | | | |
| Primordial | NA | +/– | +/– | +/– | +/– | +/– | +/– | +/– | +/– |
| Primary | NA | NA | NA | +/– | +/– | +/– | +/– | +/– | +/– |
| Secondary | NA | NA | NA | NA | +/– | +/– | + | + | + |
| Tertiary | NA | NA | NA | NA | NA | NA | NA | + | + |
| **Theca cell** | | | | | | | | | |
| Secondary | NA | NA | NA | NA | NA | NA | – | – | – |
| Tertiary | NA | NA | NA | NA | NA | NA | NA | – | – |
| **Corpus luteum** | NA | NA | NA | NA | NA | NA | NA | +/– | +/– |

+, positive; +/–, weakly positive; –, negative; and NA, not available.

**Table 2.** Details of primers for target gene amplification in pigs

| Target gene (accession number) | Primer sequence | Annealing temperature (C) | Product size (bp) |
|---|---|---|---|
| Gpr3 (HQ_606483) | F: 5′-CTTTGCTGCTGTCTTCTG-3′<br>R: 5′-GTGGTCTCTGAGTAGTAGG-3′ | 58 | 159 |
| Cyclin B (NM_001170768) | F: 5′-TGGCTAGTGCAGGTTCAG-3′<br>R: 5′-CAGTCACAAAGGCAAAGT-3′ | 58 | 199 |
| Cyclin D2 (NM_214088) | F: 5′-TTACCTGGACCGCTTCTTG-3′<br>R: 5′-GAGGCTTGATGGAGTTGTCG-3′ | 58 | 155 |
| CDK1 (NM_001159304) | F: 5′-GGGCACTCCCAATAATGAAGT-3′<br>R: 5′-GTTCTTGATACAACGTGTGGGAA-3′ | 58 | 260 |
| CDK4 (NM_001123097) | F: 5′-GCATCCCAATGTTGTCCG-3′<br>R: 5′-GGGGTGCCTTGTCCAGATA-3′ | 58 | 125 |
| GAPDH (AF_017079) | F: 5′-GGACTCATGACCACGGTCCAT-3′<br>R: 5′-TCAGATCCACAACCGACACGT-3′ | 58 | 220 |

junction (CMJ) of the ovary were initially broken down to form primordial follicles, which contained a single layer of presquamous granulosa cells (spGCs) around an individual oocyte. The oogonia and oocytes in these nests and primordial follicles displayed intense Gpr3 immunostaining, and only a small percentage of spGCs in primordial follicles exhibited faint signs of Gpr3 protein (Table 1 and Fig. 1B). In the 90-dpc ovary, primordial follicles comprised the majority of the ovarian content, although growing nests were still observed in the outermost layer of the ovarian cortex. Similar to the 70-dpc ovary, Gpr3 immunoreactivity was also faint in spGCs of primordial follicles and intense in the cytoplasm of oocytes, but it was not detected in stromal cells and the nuclei of oocytes and spGCs (Table 1 and Fig. 1C).

At 1 day post partum (dpp), almost all the egg nests were broken down, and primordial follicles were present in multiple layers in the ovarian cortex. Meanwhile, a few primary follicles with one layer of cuboidal granulosa cells (cGCs) were located at the ovarian CMJ. As displayed in Fig. 1D, Gpr3 protein was strongly expressed in the oocytes from primordial to primary follicles, weakly expressed in cGCs of primary follicles and faintly expressed in spGCs of primordial follicles (Table 1). The ovarian architecture at 25 or 35 dpp was not much different from that seen at 1 dpp. Primordial follicles were placed near the ovarian superficial cortex; many primary follicles with a single layer of cGCs were present throughout the ovarian middle cortex; and early secondary follicles with two layers of cGCs were evident at the ovarian CMJ. As shown in Fig. 1E–F, Gpr3 protein, similar to 1-dpp pigs, was also strongly stained in oocytes of primordial and primary follicles, but with lower staining in GCs. In the 70-dpp ovaries, besides primordial and primary follicles, secondary follicles containing a well-developed theca interna began to appear at the ovarian CMJ. Immunostaining for Gpr3 was mainly present in the oocytes, to a lesser extent in GCs, but was absent in the theca interna

of the follicles, including primordial, primary and secondary follicles (Table 1 and Fig. 1G). In ovaries of 140-dpp and 180-dpp pigs, the majority of the space was occupied by tertiary or Graafian follicles (Fig. 1I-K), and primordial, primary and secondary follicles were concentrated near the base of the hilar region. In addition, several corpora lutea were first observed at 180 dpp (Fig. 1L). Intense Gpr3 immunoreactivity was again evident in the oocytes from primordial to Graafian follicles (Table 1 and Fig. 1H–J). Moreover, a positive immunoreaction was observed in both cumulus and mural GCs in antral follicles (Fig. 1I–K) and was faintly present in the developing corpora lutea (Fig. 1L).

No striking differences in localization of Gpr3 protein was observed during the gestation, growth and development period investigated. In addition, no signal was observed in sections incubated with NGS instead of primary antibody as a negative control (Fig. 1M–N), while a strong signal was detected in the section of porcine cerebral cortex incubated with Gpr3 antibody as a positive control (Fig. 1O). Relative levels of immunostaining were evaluated and determined at least four times.

*Expressional levels of Gpr3 mRNA and protein in ovaries of different ages of pigs*

To investigate the developmental changes in Gpr3 mRNA and protein in the ovaries of pigs, multiage ovaries, including 1, 25, 35, 70, 140 and 180 days post partum, were used for real-time RT-PCR and Western blotting. *GAPDH* was used as an endogenous reference for determination of *Gpr3* mRNA profiles. As displayed in Fig. 2A, we found that the transcript for *Gpr3* was detected in all the porcine ovaries at different ages, but there were wavy changes in its expression levels from Day 1 to Day 180 post partum (dpp). The *Gpr3* mRNA expression level in the 1-dpp ovary was the highest among all the ages and was significantly downregulated until 70 dpp. However, a temporary increase occurred at 140 dpp ($P<0.01$) followed by a drop at 180 dpp ($P<0.01$). In addition, the relative mRNA expression levels of *Gpr3* showed significantly negative correlation ($r = –0.513$, $P<0.05$) with the age from birth to sexual maturity (180 dpp) of pigs. The quantification of protein level was carried out by Western blotting, and β-actin served as an internal control. The specificity of the Gpr3 antibody was evaluated in our previous studies by the molecular weight of Gpr3 protein [22]. In this study, a clear band for Gpr3 at all the developmental stages was observed at ~35 kD (Fig. 2B). Moreover, the Gpr3 protein expression pattern was similar to that of its mRNA during the developmental stages (Fig. 2B). The Gpr3 protein decreased continuously from 1 to 35 dpp ($P<0.05$), specifically from 1 to 25 dpp ($P <0.01$), and then remained at low levels until 70 dpp. Subsequently, a significant increase was observed at 140 dpp ($P<0.01$). After the increase, the Gpr3 protein level was downregulated again at 180 dpp ($P<0.01$), but its level was significantly higher than that at 25, 35 and 70 dpp ($P<0.01$). These data suggest that the expression of Gpr3 receptor exhibits a specific developmental pattern in the porcine ovary.

*Expressional levels of Gpr3 mRNA and protein in follicles with different diameters*

In order to investigate the expression pattern of Gpr3 receptor in different developmental stage follicles, multisized follicles, including

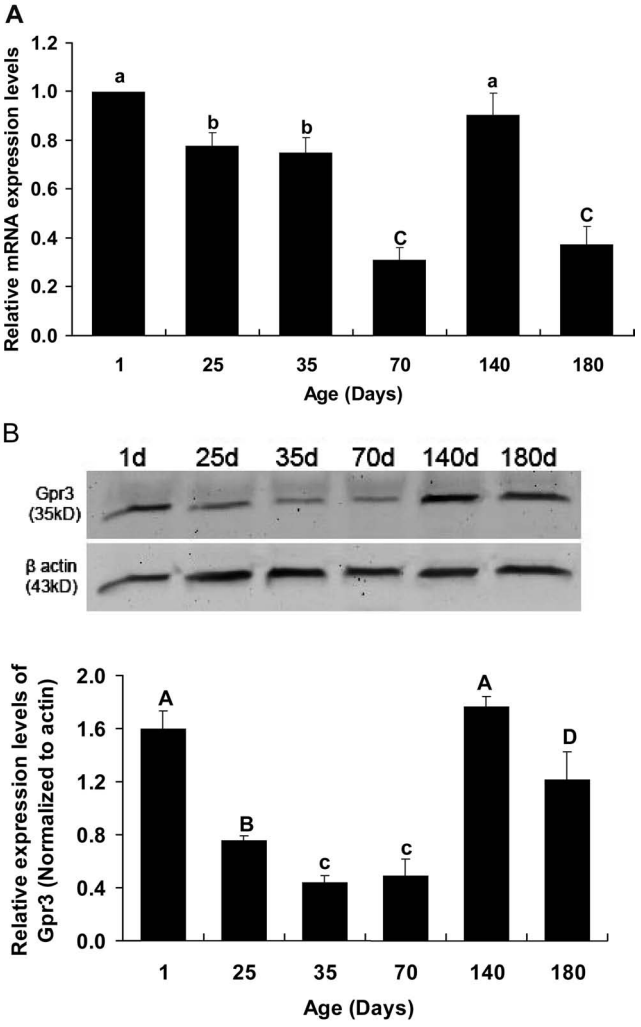


**Fig. 2.** Developmental changes in the relative abundance of mRNA (A: real-time PCR data) and protein (B: Western blotting data) expression of Gpr3 in porcine ovaries at different ages. Bars with different lowercase letters indicate significant differences ($P<0.05$), while bars with different uppercase letters indicate significant differences ($P<0.01$). The graph shows means ± SD. Experiments were repeated at least three times. (A): The mean value in the control (1 dpp) group was set as 1.

small (< 3 mm), medium (3–5 mm) and large (> 5 mm) ones, were grouped on the basis of their diameters for real-time RT-PCR and Western blotting assays. Gpr3 mRNA and protein expression were both evident in different sized follicles (Fig. 3). As displayed in Fig. 3A, the lowest mRNA expression of *Gpr3* was found in small follicles ($P<0.01$), and the expression increased ($P<0.01$) during follicle growth. Furthermore, like its mRNA, Gpr3 protein levels were also directly related ($P<0.01$) with follicle diameter, since they significantly increased during follicle growth (Fig. 3B).

*Effect of siRNA on Gpr3 expression*

We knocked down *Gpr3* mRNA in porcine GCs by siRNA. The expression of Gpr3 mRNA and protein was analyzed by real-time

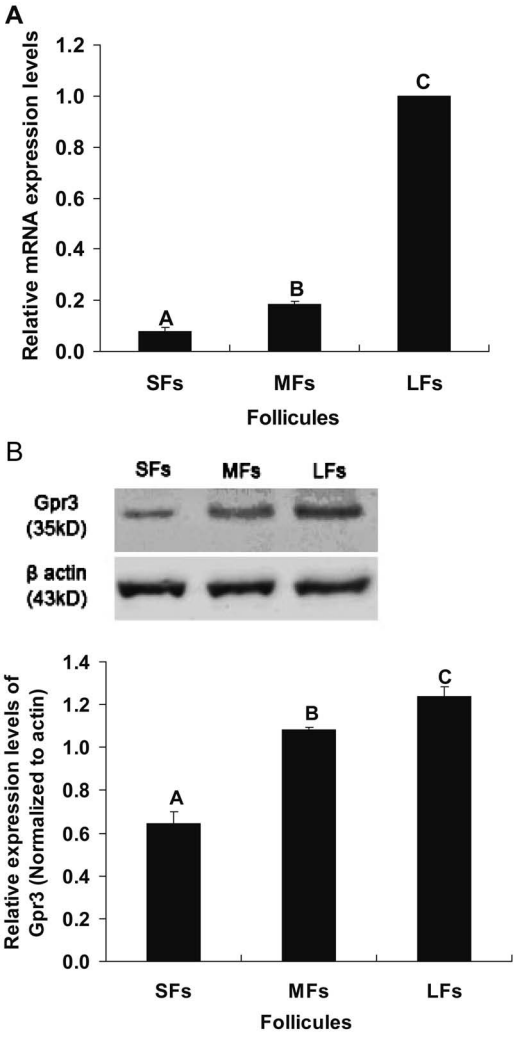


**Fig. 3.** The relative abundance of Gpr3 mRNA (A: real-time PCR data) and protein (B: Western blotting data) expression in different diameter follicles. Bars with different uppercase letters indicate very significant differences (P<0.01). The graph shows means ± SD. Experiments were repeated at least three times. SFs, small follicles; MFs, medium follicles; LFs, large follicles (A): The mean value in the control (LFs) group was set as 1.

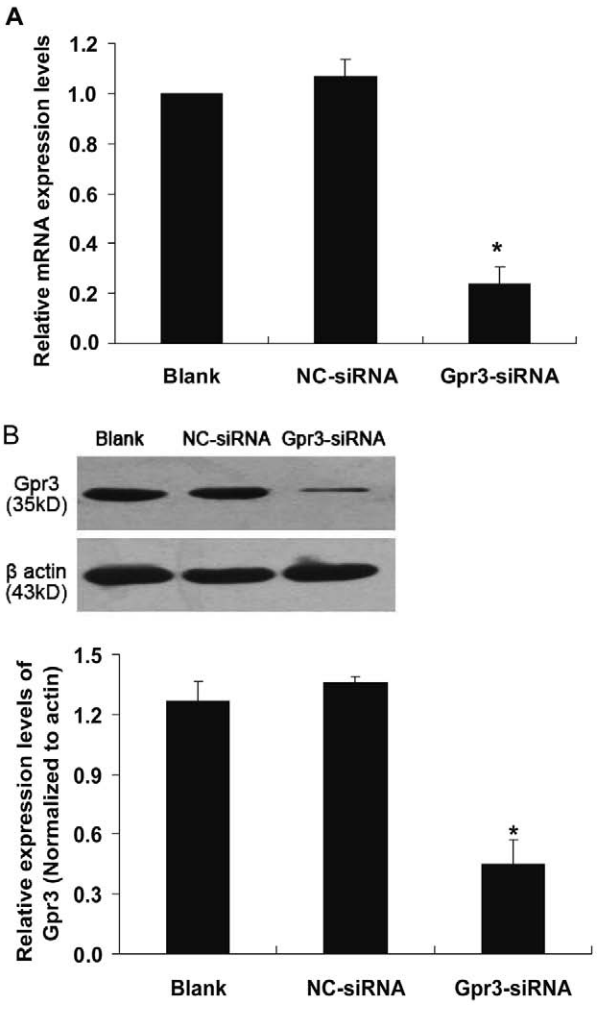


**Fig. 4.** The relative abundance of Gpr3 mRNA (A: real-time PCR data) and protein (B: Western blotting data) expression in GCs after transfection for 48 h. Statistically significant differences from the blank group are indicated by an asterisk (P<0.05). The graph shows means ± SD. Experiments were repeated at least three times. (A): The mean value in the control (Blank) group was set as 1.

RT-PCR and Western blotting post transfection, as displayed in Fig. 4. The results in Fig. 4A show that the expression of Gpr3 mRNA was inhibited after transfection with Gpr3-siRNA, with the Gpr3 transcript level being reduced by approximately 73% compared with that in the control cultures (blank and NC-siRNA). Western blotting analysis of Gpr3 expression clearly revealed the suppressive effect of Gpr3-siRNA on the expression of the Gpr3 protein in the GCs (Fig. 4B). The results demonstrated that the level of Gpr3 protein also markedly decreased 48 h post transfection with the Gpr3-siRNA, by approximately 64.4% (Fig. 4B).

*Effect of Gpr3 knockdown on cell proliferation of GCs*

Cell proliferation was measured using the MTT assay. As shown in Table 3, transfection of NC-siRNA did not significantly change the cell proliferation of GCs as compared with the blank group (P>0.05), suggesting that the transfection process itself did not affect the cell proliferation assay. However, Gpr3 knockdown significantly promoted the growth of GCs, as compared with the control culture (blank and NC-siRNA) cells (Table 3).

*Effect of Gpr3 knockdown on the cell cycle of GCs*

To further determine the effect of Gpr3 knockdown on the proliferation of GCs, flow cytometry was used to analyze the cell cycle changes of GCs. The results in Table 4 show that Gpr3 knockdown could significantly decrease the proportion of cells in the G0/G1 phase and increased the cells in the S phase, but there were no significant differences in the proportions of cells in the G2 phase.

**Table 3.** Effect of Gpr3 knockdown on cell proliferation of GCs

| Group | OD 550 |
|---|---|
| Blank | $0.514 \pm 0.059^{a}$ |
| NC-siRNA | $0.503 \pm 0.057^{a}$ |
| Gpr3-siRNA | $0.689 \pm 0.076^{b}$ |

Values are presented as means ± SD. Statistically significant differences (P<0.05) among the groups are indicated by different letters.

**Table 4.** Effect of Gpr3 knockdown on cell cycle of GCs

| Group | Cell cycle | | |
|---|---|---|---|
| | G0/G1 (%) | S (%) | G2/M (%) |
| Blank | $91.71 \pm 0.23^{a}$ | $5.28 \pm 0.31^{a}$ | $3.01 \pm 0.15^{a}$ |
| NC-siRNA | $91.45 \pm 0.72^{a}$ | $5.39 \pm 0.44^{a}$ | $3.16 \pm 0.66^{a}$ |
| Gpr3-siRNA | $90.24 \pm 0.69^{b}$ | $6.70 \pm 0.72^{b}$ | $3.05 \pm 0.11^{a}$ |

Values are means ± SD. Significant differences among the groups are indicated by different letters (P<0.05).

*Effect of Gpr3 knockdown on cell cycle marker genes of GCs*

To investigate the mechanism by which Gpr3 knockdown regulates the cell cycle of GCs, we measured the expression levels of Cyclin B1, Cyclin D2, CDK1 and CDK4, functional markers of cell proliferation (Fig. 5). In Gpr3 knockdown cells, Cyclin B1 and Cyclin D2 mRNA levels were significantly higher than those in the controls (blank and NC-siRNA) (P<0.05). However, CDK1 and CDK4 mRNA levels showed no significant differences between the three groups (P>0.05).

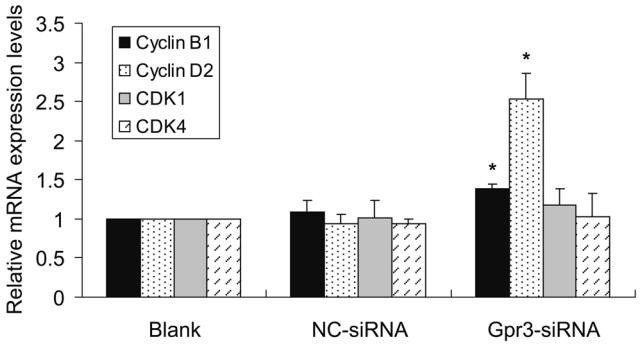


**Fig. 5.** Effect of Gpr3 knockdown on Cyclin B1, Cyclin D2, CDK1 and CDK4 mRNA levels in GCs. An asterisk indicates statistically significant differences (P<0.05) among the groups. The graph shows means ± SD. Experiments were repeated at least three times. The mean value in the control (Blank) group was set as 1.

## Discussion

RNA encoding *Gpr3* has been known to be expressed in the mouse ovary for nearly twenty years [12], but its breeding-related feature was discovered only in the last few years. In this study, to examine the relationship between the Gpr3 receptor and ovarian development, we investigated the temporal and spatial localization of porcine Gpr3 in the ovary during fetal and postnatal development. Immunohistochemical analysis showed that Gpr3 protein was present in multiage ovaries, notably expressed in the oocyte and weakly expressed in GCs and cumulus cells but was not detected in the ovarian surface epithelium and the theca cells of antral follicles, which is similar to its mRNA distribution in mice [15, 17]. However, Hinckley *et al.* reported that *Gpr3* mRNA was only detected in mouse cumulus cells, rather than in mural GCs [25], suggesting that species-specific expression of Gpr3 might exist in the mammalian ovary. Moreover, the staining intensity of Gpr3 in the GCs of the secondary follicle stage onwards was stronger than those of primordial and primary follicles. Previous studies have also shown that the complex bidirectional communication between the oocyte and the somatic compartments of the follicle becames more apparent after the emergence of secondary follicles [7]. Because Gpr3 was expressed in the GCs, albeit at low levels, it was possible that the GCs could produce cAMP and provide it to the oocyte for regulation of meiosis through gap junctions that are present between the oocyte and somatic cells [19]. In addition, Mehlmann *et al.* [15] reported that the Gpr3 receptor was a link in communication between somatic cells and the oocyte of the ovarian follicle and was crucial for the regulation of meiosis. Therefore, we could presume that Gpr3 protein might function through the oocyte pathway in the early stage of follicular development but function through the interaction between the oocyte and granulosa cell pathway in terminal follicular growth. Interestingly, the Gpr3 protein was also detected in the oogonia, oocyte and intercellular bridge of egg nests in the fetal ovary, suggesting that Gpr3 not only played a role in maintaining meiotic arrest of the oocyte [15] but also might be involved in germ cell cluster breakdown and primordial follicle endowment. In addition, a Gpr3 signal was also found in the corpora lutea, which showed that Gpr3 might play a role in signal pathways in luteal cells. Together, the stage- and cell-specific expressional patterns of Gpr3 protein in the ovary suggested that Gpr3 protein might play potential roles in follicular development and luteinization in pigs.

In order to further understand the expression pattern of the porcine Gpr3 receptor, we investigated Gpr3 expression in the ovary including follicles at both mRNA and protein levels, and the results indicated that a similar expression pattern of Gpr3 was present in them. In the multiage ovaries, Gpr3 mRNA and protein were both detected, corresponding to the results of immunohistochemical analysis, and the expression levels were developmentally regulated. Interestingly, the peak value of the Gpr3 expression was observed at around 140 dpp; however, the reason for this requies further investigation. When the expression pattern of Gpr3 was investigated in multisize follicles and COCs during IVM [22], we found a different expression tendency between them. The Gpr3 expression level in multisize follicles was upregulated significantly during follicle growth, while a significant decrease was present in the whole COCs during IVM [22]. Previous studies have shown that mammalian oocytes could undergo spontaneous resumption of meiosis independently of gonadotropins upon their removal from the ovarian follicle [20], suggesting that the ovarian follicle provides an inhibitory environment that maintains the enclosed oocyte at meiotic arrest. Gpr3, which was found in the follicle, was reported to play an important role in the maintenance

of meiotic prophase arrest in the process of mammalian oocyte maturation. *In vivo*, upregulation of Gpr3 expression during follicle growth was present to promote meiotic arrest in the oocyte. However, a significant decrease in Gpr3 expression was present in whole COCs removed from follicles during IVM. These results indicated that some unknown mechanisms might exist in the follicle that promote Gpr3 receptor expression during follicle growth to maintain the meiotic arrest of the oocyte. LH from the pituitary acts on the ovarian follicle to cause resumption of meiosis in the oocyte, as well as ovulation [8]. It was thought that there was a possible pathway by which LH action on somatic cells decreases the production of cAMP by inhibition of Gpr3-Gs-AC signaling, but Rachael *et al.* [26] reported that LH did not cause meiotic resumption by terminating Gpr3-Gs signaling. Moreover, the Gpr3 protein was still detected in the preovulatory follicle and corpora lutea, suggesting that LH really did not terminate the Gpr3 signaling. In addition, recent studies showed that Cyclic GMP from the surrounding somatic cells had an ability to maintain meiotic arrest in mouse oocytes through inhibition of the hydrolysis of cAMP by the phosphodiesterase PDE3A. Moreover, the inhibitory signal can be reversed by LH through lowering of the cGMP levels in somatic cells and closing gap junctions between the somatic cells [27]. The granulosa cell ligand NPPC and its receptor NPR2 were also considered to be an important system to prevent precocious meiotic maturation in mouse oocytes, which is critical for maturation and ovulation synchrony and for normal female fertility [28]. However, how LH signaling leads to the resumption of meiosis in the oocyte needs further studies.

To further understand the effect of the endogenous Gpr3 receptor on porcine GCs, RNAi was employed to silence the Gpr3 gene. It was observed that knockdown of the endogenous Gpr3 gene blocked Gpr3 signaling, as demonstrated by real-time RT-PCR and Western blotting. Moreover, the MTT analysis showed that knockdown of the endogenous Gpr3 gene promoted the proliferation of GCs *in vitro*, suggesting that the porcine Gpr3 might play roles in proliferation of GCs during follicular development. It has been well documented that cell proliferation depends on the cell cycle. Therefore, we investigated the effects of knockdown of Gpr3 on the cell cycle of porcine GCs. The results shown that knockdown of Gpr3 significantly decreased the proportion of cells in the G0/G1 phase while significantly increasing the cells in the S phase. The G0/G1 checkpoint can be viewed as a master checkpoint of the mammalian cell cycle [29, 30]. Regulation of the G0/G1 phase of the cell cycle involves many different families of cyclins and cyclin-dependent kinases (CDKs). In this study, enhancement of proliferation of GCs by knockdown of Gpr3 was associated with an increase in Cyclin B1 expression. Cyclin B1 acts as a downstream gene of Gpr3, participating in the regulation of meiotic arrest in *Xenopus laevis* oocytes [31], which suggests that it might be involved in regulating cell proliferation in porcine GCs. Interestingly, the Cyclin D2 expression level also increases after knockdown of Gpr3 in GCs, and there has been no previous report about the relationship between Gpr3 and Cyclin D2. How Gpr3 regulates the expression of Cyclin D2 needs further studies. RNAi-mediated Gpr3 silencing resulted in increased proliferation of the cells and an increased proportion of cells in the S phase, indicating that Gpr3 signaling might play a role in the normal growth of porcine GCs during follicular development.

In conclusion, our results revealed the temporal and spatial localization of porcine Gpr3 during fetal and postnatal development in the ovary of pigs, its expression pattern in multiage ovaries and multisize follicles, and its function in proliferation of GCs *in vitro*. The stage- and cell-specific expressional pattern in the ovary suggested that Gpr3 might play an important role during the entire follicular development and luteinization in pigs. Knockdown of the endogenous Gpr3 gene promoted cell proliferation of GCs *in vitro*, suggesting that the Gpr3 might play a role in normal proliferation of porcine GCs during follicular development. All the above results provided a basis for further research of the porcine Gpr3 gene.

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