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Original article

Expression of follicle-stimulating hormone receptor (FSHR), protein kinase B-2 (AKT2) and adapter protein with PH domain, PTB domain, and leucine zipper (APPL1) in pig ovaries

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Abstract

Follicle-stimulating hormone (FSH) regulates oogenesis and spermatogenesis by binding to its receptor (FSHR) on target cells in the ovary and testis, respectively. The signaling cascades activated after ligand binding are extremely complex and have been shown to include protein kinase A and phosphatidylinositol 3-kinase/protein kinase. The adapter protein APPL1 (adapter protein with PH domain, PTB domain, and leucine zipper), which is an assortment of other signaling proteins, was previously identified to interact with the FSH receptor (FSHR) and the protein kinase B (AKT) pathway. APPL1 plays an important role in promoting cell survival within the preovulatory follicle granulosa layer. Here, we aimed to evaluate the *FSHR*, *AKT2,* and *APPL1* gene and protein expression levels in the ovaries of different prolific porcine breeds (Wannan Black [WB] and Large White [LW] pigs) using immunohistochemistry and qRT-PCR, respectively. Our results showed that *FSHR, AKT2,* and *APPL1* mRNA levels were significantly higher ($P < 0.05$) in the ovaries of WB pigs than in the ovaries of LW pigs. Additionally, the FSHR, AKT2, and APPL1 proteins were mainly found distributed in the granulosa cells and oocytes. This study showed that high levels of FSHR, AKT2, and APPL1 were expressed in the ovaries of high prolific breed pigs.

Key words: pigs, immunohistochemistry, FSHR, AKT2, APPL1

Introduction

Porcine fertility is one of the most significant economic traits in pig production as it is directly related to production efficiency. Previous research has shown that follicle-stimulating hormone (FSH), which is secreted from the pituitary, is central to reproduction in mammals as it interacts with FSH receptor (FSHR) that is mainly located by the ovary (Simoni et al. 1997, Robker et al. 1998, Dias et al. 2002). The mechan-

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ism that regulate the development of follicles is a complex process and it is mainly regulated by FSH (Guthrie 2004). The phosphoinositide 3-kinase (PI3K)/AKT (protein kinase B) pathway is a significant signaling corridor necessary for the FSH signal because it leads to phosphorylation and activation of the nodal kinase AKT (protein kinase B) (Zeleznik et al. 2003). Previous studies have demonstrated that the AKT pathway plays an important role in promoting cell survival within the preovulatory follicle granulosa layer (Johnson et al. 2001).

APPL1 (adapter protein with PH domain, PTB domain, and leucine zipper) has been shown to interact with many signaling proteins and receptors, for instance, APPL1 interacts with the p110a catalytic subunit of PI3K and inactive AKT (Mitsuuchi et al. 1999). However, the study suggested that APPL1 was dispensable for development and only participated in AKT signaling under certain conditions (Nechamen et al. 2007). Previous studies have demonstrated that APPL1 participates in the FSH-induced IP_3 pathway, implicating the role of APPL1 in intracellular calcium signaling (Thomas et al. 2011). FSHR-positive staining was observed in the oocytes of primordial and granulosa cells of primary follicles (Durlej et al. 2011). In pigs, FSHR expression has been mainly studied in the pre-antral and antral follicles (Cardenas et al. 2002). In another study, *FSHR* gene expression was upregulated in the granulosa cells of the ovary (An et al. 2009).

Despite these data, little information is available on the expression and distribution of FSHR, AKT2, and APPL1 in porcine ovaries. Thus, in this study, we aimed to explore the *FSHR, AKT2* and *APPL1* mRNA expression levels and distribution of the FSHR, AKT2, and APPL1 proteins in porcine ovaries. We believe that the findings of this study will serve as a foundation for further studies on the relationship between the three genes and reproduction in pigs.

Materials and Methods

Animals and sample collection

For this study, we obtained a total of 12 sows aged 5-6 years, including six Wannan Black pigs (WB) from the breeding farm in the Jixi county of Anhui province; three Large White pigs (LW) purchased from the Antai Swine Breeding Farm in Feidong County, Hefei City of Anhui province; and three LW pigs that had been supplied by the Da Ziran Swine Breeding Farm in Huaibei City of Anhui province. At slaughter, all the left ovaries were obtained and immediately fixed in 4% paraformaldehyde solution for 24 hours for immunohistochemical localization of FSHR, APPL1, and AKT2. All the right ovary samples were cut into small pieces, immediately frozen in liquid nitrogen, and stored at -80°C until gene analysis. All the procedures involving animals were approved by the Animal Care and Use Committee of Anhui Agricultural University.

Immunohistochemistry

The left ovary samples were cut into approximately 1-cm^3 sections and fixed in paraformaldehyde for 24 h. The samples were then transferred to 75% ethanol and incubated overnight, dehydrated in a graded series of ethanol (80% ethanol for 2 h, 95% ethanol for 2 h, and 100% ethanol for 2 h), cleared with xylene, and embedded in paraffin. The paraffin blocks were cut into approximately 5- μm-thick sections using a rotary microtome (Leica RM-2235; Leica Microsystem Inc., Bensheim, Germany).

After dewaxing, the samples were washed thrice with 0.1 M phosphate-buffered saline (PBS) for 5 min each time. Then, tissue samples were boiled in 0.01% citrate buffer in a microwave oven (800 W for 10 min) to retrieve antigenicity. After cooling, the tissue paraffin blocks were immersed in 3% H_2O_2 for 10 min at room temperature to remove endogenous peroxidase activity. The samples were washed thrice with PBS (5 min), blocked with 5% bovine serum albumin (BSA, Sigma, V900933), and separately reacted with rabbit anti-FSHR antibodies (1:75 dilution, Bioss Biological Technology Company, bs-20658R), rabbit anti-AKT2 antibodies (1:100 dilution, Bioss Biological Technology Company, bs-10724R), and rabbit anti-APPL1 antibodies (1:100 dilution, Bioss Biological Technology Company, bs-1632R) in a moist chamber overnight at 4°C. In all reactions, negative controls were run in parallel by omitting the mixture of primary antisera (substituted by PBS) to demonstrate the absence of specific immunoreactivity. After rinsing with PBS, the samples were incubated for 15 min at 37°C with goat anti-rabbit IgG, besides, DAB was used to visualize the antigens. Then, the samples were lightly counterstained with Mayer's hematoxylin, dehydrated, placed on a cover slip, and examined under a light microscope. The negative control included three antibodies were replaced with 1% BSA. The pre-absorption control for the APPL1, AKT2 and FSHR primary antibodies used here were validated by immunohistochemical staining with a well-characterized respective antiserum.

Table 1. Primers and size of the amplification products of the target gene in the real-time polymerase chain reaction (RT-PCR).

Gene	Accession number	Primer sequence (5'-3')	Product size (bp)
β-*actin*	U07786.1	F-CTCGATCATGAAGTGCGACGT S-GTGATCTCCTTCTGCATCCTGTC	114
FSHR	NM_214386	F-TCGAGGCAAATGTGTTCTCC S-AAGGTTCTGGAAGGCATCAG	101
AKT2	NM_001256779.1	F-TTCTTCCTCACCGTCAACTGG S-GGCGTGATTGTGATGGATTGG	134
APPL1	NM_001134966.1	F-ACTGCTAAGGACAACAAACTGC S-ACTGCTAAGGACAACAAACTGC	150

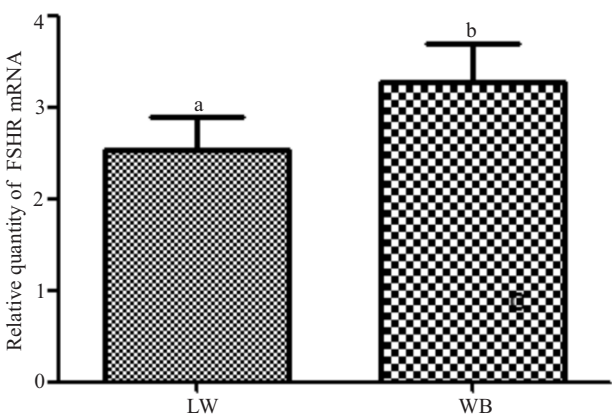

Fig. 1. Expression levels of *FSHR* mRNA in the ovaries of Large White (LW) and Wannan Black (WB) pigs. Data are presented as means ± standard error. Different letters indicate a significant difference ($p<0.05$) between the LW and WB pigs.

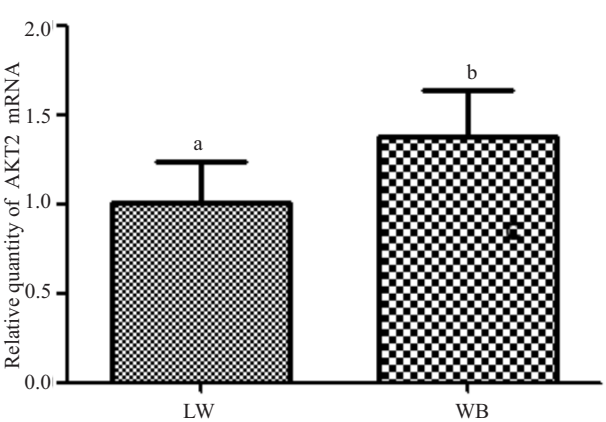

Fig. 2. Expression levels of *AKT2* mRNA in the ovaries of Large White (LW) and Wannan Black (WB) pigs. Data are presented as means ± standard error. Different letters indicate a significant difference ($p<0.05$) between the LW and WB pigs.

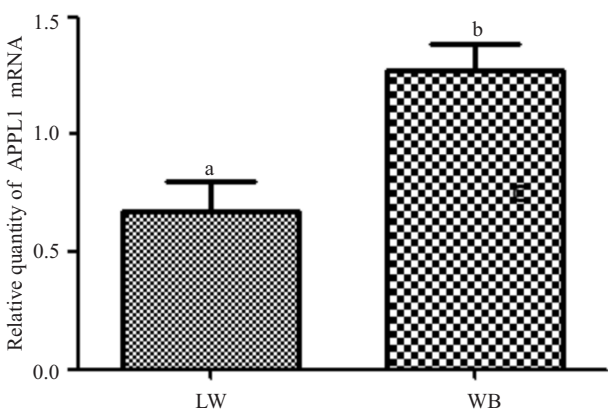

Fig. 3. Expression levels of *APPL1* mRNA in the ovaries of Large White (LW) and Wannan Black (WB) pigs. Data are presented as means ± standard error. Different letters indicate a significant difference ($p<0.05$) between the LW and WB pigs.

qRT-PCR procedure and statistical analyses

The total RNA was extracted from all right ovary samples with the EZNA total RNA Kit II (Omega Bio-tek, USA), and stored at -80°C until cDNA synthesis. The RNA quality was checked by 1% agarose gel electrophoresis, and stained with 0.5 μg/mL ethidium bromide. Approximately 1 μg of RNA was reverse transcribed into cDNA in a total volume of 20 μL using the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, RR047A, Japan).

The amplified cDNA of the *FSHR, AKT2,* and *APPL1* genes was investigated. The β-actin gene was chosen as a reference gene. All primer sequences (Table 1) were designed using Primer 5.0. All PCR experiments were performed with CFX96 Real-Time PCR Detection System (Bio-Rad, USA), and using SYBR Green PCR Master Mix (Applied Biosystems)

Table 2. Pearson's correlation of mRNA expression of various genes.

	FSHR mRNA	FSHR mRNA	APPL1 mRNA
FSHR mRNA	×	0.981*	0.487
AKT2 mRNA	0.981*	×	0.626
APPL1 mRNA	0.487	0.626	×

* indicates $p<0.05$.

according to the manufacturer's protocol. In this study, 1 μL of cDNA was added to a 19- μL mixture containing 10 μL iTaq™ Universal SYBR Green Supermix (Bio-Rad, USA), 1 μL of each primer, and 7 μL of RNase-free H_2O. The reaction was amplified at 95°C for 3 min, followed by 40 cycles of 95°C for 30 s and 61.5°C for 30 s. For each sample, three replications were performed, and all reactions were run in triplicate.

The cycle threshold was collected from each reaction, and the relative expression level of different genes mRNA to β-actin mRNA was evaluated using the $2^{-\Delta\Delta CT}$ method (Hellemans et al. 2007). Data were analyzed by the *t* test and reported as the means ± SEM. Statistical analysis was performed using SPSS version 19.0. A *p* value of <0.05 was considered statistically significant.

Results

Expression of *FSHR*, *AKT2*, and *APPL1* mRNA in the ovary

The results of the qRT-PCR revealed that the *FSHR* (Fig. 1), *AKT2* (Fig. 2) and *APPL1* (Fig. 3) mRNA expression levels in the ovary were significantly higher ($p<0.05$) in WB pigs than in LW pigs (Fig. 1). Furthermore, *FSHR* mRNA expression had a significantly positive correlation with *AKT2* mRNA expression (Table 2).

Distribution of FSHR, AKT2, and APPL1

The immunohistochemical analyses were performend to revealed the distribution of protein expressions. In LW and WB pigs, No immunoreactivity was found for FSHR (Fig. 4A,D), AKT (Fig. 5A,D) or APPL1 (Fig. 6A,D) with the omission of the respective primary antibodies. FSHR were mainly located in the granulosa cells and oocytes of secondary and primary and strong immunoreactivity was observed in WB pigs (Fig. 4B,C,E,F). In primordial follicle of LW and WB pigs, weak immunoreactivity was observed indicating the possibility that the effect of FSHR on follicle was based on granulosa cells. The distribution of AKT (Fig. 5 B,C,E,F) and APPL1 (Fig. 6 B,C,E,F) were similar to FSHR which were mainly expressed in the granulosa cells and oocytes of secondary and primary follicles and the immunoreactivity of WB pigs is stronger than LW pigs.

Discussion

We found that FSHR, APPL1, and AKT2 proteins and genes were all expressed in pig ovaries and mainly distributed in the granulosa cells and oocytes of secondary and primary follicles, weak express was found in primordial follicle. The FSHR, APPL1, and AKT2 expression levels in the ovaries were all higher in the WB pigs than in the LW pigs. A similar result was reported in the study of Cardenas et al. (Johnson et al. 2001, Cardenas et al. 2002). The WB pigs belonged to a Chinese native breed with higher reproduction performance than LW pigs. It is known that FSHR plays an important role when FSH stimulate the development of follicle. A previous study in Chinese alligators found changes in the FSHR expression, suggesting that the FSHR plays an important role in promoting ovarian development during the female reproductive cycle (Durlej et al. 2011). Our study found that the FSHR was not only expressed in granulosa cells but also in oocytes, indicating the potential role of FSH in the modulation of meiotic resumption and completion of oocyte maturation (Meduri et al. 2002, Patsoula et al. 2003).

Previous studies have demonstrated that the AKT pathway plays an important role in promoting cell survival within the preovulatory follicle granulosa layer via FSHR (Johnson et al. 2001). The PI3K pathways is a crucial pathway by which FSH signals lead to the phosphorylation and activation of the nodal kinase AKT (protein kinase B). Therefore, the AKT pathway was suggested to play an important role in reproduction, and it was previously shown that AKT activity plays an important role in directing endometrial cell fate during pregnancy and that the PI3K/AKT pathway might be involved in the endometrium (Veillette

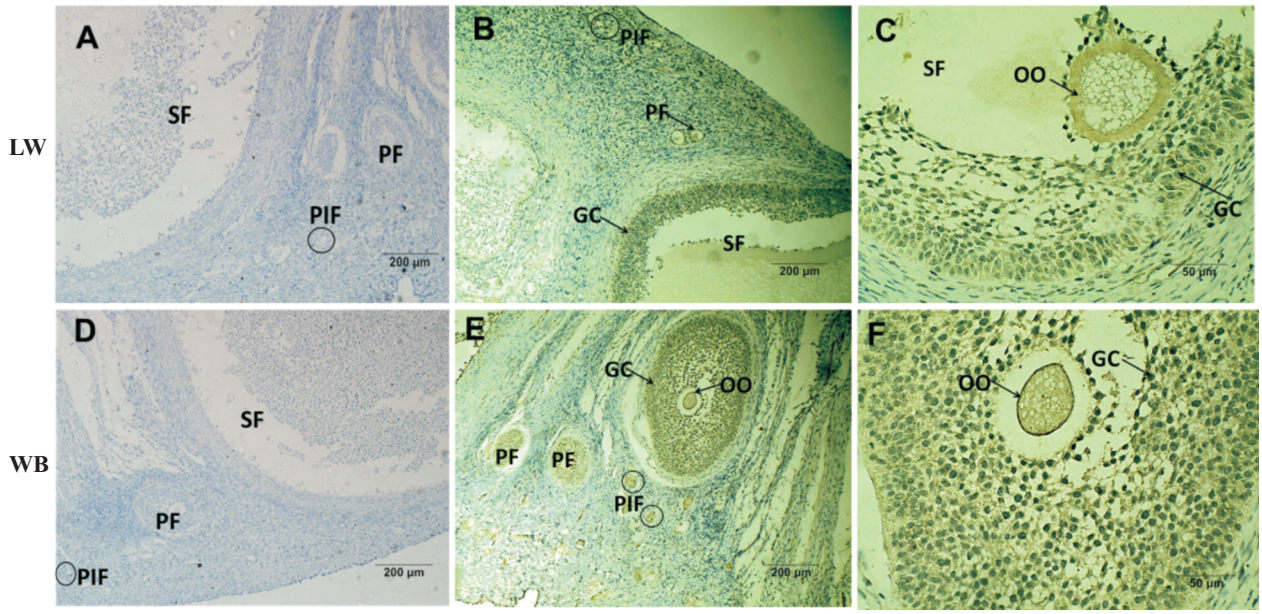

Fig. 4 Distribution of FSHR in the different follicles of two group. A, D: Negative control, B,C: Distribution of FSHR in Large White pigs; E, F: Distribution of FSHR in Wannan Black pigs. LW: Large White pigs, WB: Wannan Black pigs. SF: Secondary follicle, PF: primary follicle, PIF: primordial follicle, GC: granulosa cells, OO: oocyte.

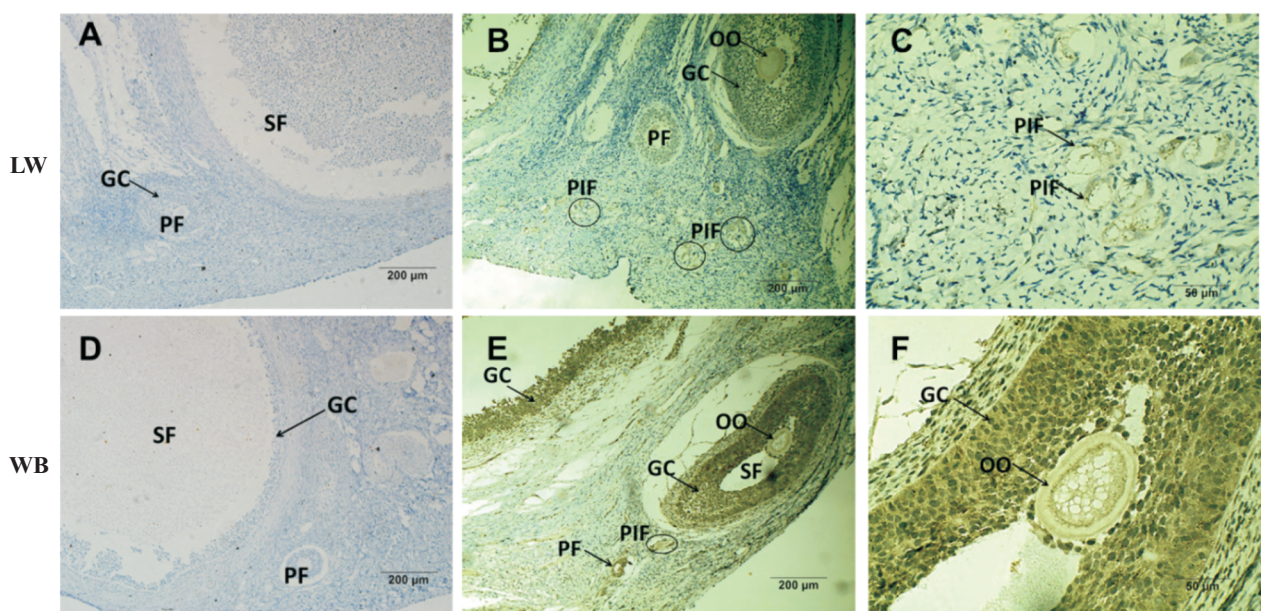

Fig. 5 Distribution of AKT in the different follicles of two group. A, D: Negative control, B,C: Distribution of AKT in Large White pigs; E, F: Distribution of AKT in Wannan Black pigs. LW: Large White pigs, WB: Wannan Black pigs. SF: Secondary follicle, PF: primary follicle, PIF: primordial follicle, GC: granulosa cells, OO: oocyte.

et al. 2013). Results of research in chickens support the role of an active PI3-kinase/AKT signaling in the maintenance of the preovulatory follicle granulosa layer (Johnson et al. 2001). Additionally, other researchers have identified a pathway by which FSH-stimulated PKA hijacks GAB2 and IRS-1 to activate the PI3K pathway in granulosa cells, leading to enhanced proliferation, inhibition of apoptosis, enhanced translation, and activation of target genes with products that define the mature, preovulatory GC (Hunzicker-Dunn et al. 2012, Wang et al. 2012).

Nechamen et al. identified the association between *APPL1* and *FSHR* (Nechamen et al. 2004). In a later study, Nechamen et al. suggested that APPL1 plays key roles in regulating PI3K/AKT signaling (Nechamen et al. 2007). These findings are in agreement with the results of another study, which found that APPL1 participates in the FSH-induced IP_3 pathway and was

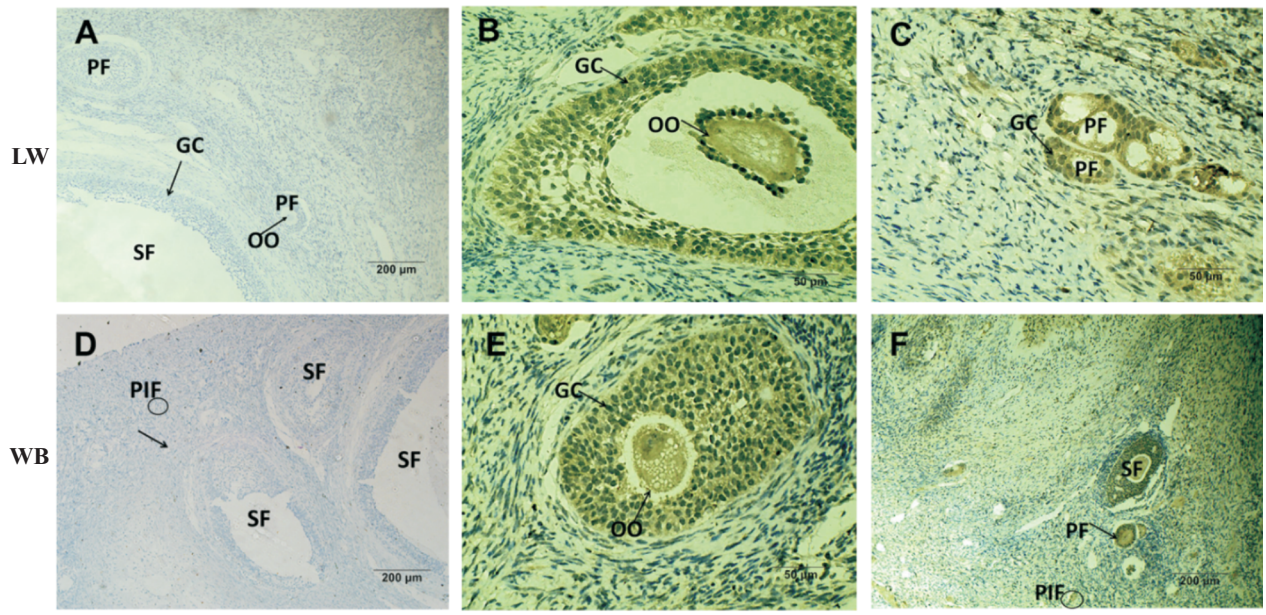

Fig. 6 Distribution of APPL1 in the different follicles of two group. A, D: Negative control, B,C: Distribution of APPL1 in Large White pigs; E, F: Distribution of APPL1 in Wannan Black pigs. LW: Large White pigs, WB: Wannan Black pigs. SF: Secondary follicle, PF: primary follicle, PIF: primordial follicle, GC: granulosa cells, OO: oocyte.

implicated in intracellular calcium signaling, confirming its interaction with FSHR (Thomas et al. 2011).

Conclusion

The immunohistochemical results of the present study confirm that FSHR, AKT2, and APPL1 presente in the porcine ovary. The RT-PCR results indicated that the *FSHR*, *AKT2*, and *APPL1* mRNA expression levels in the ovary are higher in WB pigs than in LW pigs, suggesting that these three genes may participate in the development of follicle.

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