—Full Paper—

Cellular Localization of Inhibin α -subunit, PKB/Akt and FoxO3a Proteins in the Ovaries of Minipigs

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Abstract. Experiments were conducted to examine the cellular localization of inhibin α -subunit, protein kinase B (PKB/Akt), and FoxO3a proteins in the ovaries of minipigs, Chinese Xiang pigs, by immunohistochemistry. The results indicated that inhibin α -subunits were localized in the granulosa cells of follicles at all stages but were not localized in corpora lutea. PKB was localized in the granulosa cells of primordial follicles and in the basal layers of the granulosa cells of preantral and antral follicles, but were not localized in atretic follicles and corpora lutea. FoxO3a was localized in the granulosa cells of follicles at all stages and was extensively localized in the cytoplasm of the luteinized granulosa cells of corpora lutea. Together, the stage- and cell-specific expression patterns of inhibin α -subunit, FoxO3a, and PKB suggest that these proteins might play potential roles in follicular development, atresia, and luteinization in the minipig.

Key words: FoxO3a, Inhibin, Minipig, Ovary, Protein kinase B (PKB)

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Xiang pigs are a world famous breed of miniature pigs mainly distributed in the Guizhou and Guangxi provinces of China. The miniature pig has become a perfect animal model for biomedical research because of its dwarfism, purity gene type, physiological characteristics similar to human beings and lack of inbreeding degradation [1–5]. However, knowledge of the reproductive physiology of this species is extremely limited.

Ovarian inhibin is a key hormone that regulates the development, selection, and dominance of follicles through suppression of pituitary follicle-stimulating hormone (FSH) secretion [6–8]. Active and passive immunization against inhibin is a good way to induce superovulation in many animals through an increase in the concentration of FSH [8–16]. Ovarian inhibin is composed of disulfide-bonded α - and β -subunits. Combination of the α subunit with either the βA or βB subunit ($\alpha/\beta A$ or $\alpha/\beta B$) forms an inhibin molecule. Although a limited number of studies have described the immunohistochemical localization of inhibin in

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pigs [17, 18], there is still no data on their immunohistochemical localization in the ovaries of minipigs.

Protein kinase B (PKB, also called Akt) is a serine/threonine protein kinase that functions as a critical regulator of nutrient metabolism, cell growth, proliferation, and apoptosis [19, 20]. PKB can be phosphorylated and activated by FSH in rat granulosa cells via the protein kinase-A independent pathway [21]. Although there are several reports about localization of PKB in different tissues and cells [22, 23] and immunoblot analysis of PKB in ovaries [24], the immunolocalization of PKB in the ovary has yet to be reported.

Forkhead members of the O class (FoxO) are transcription factors that play a critical role in a growing number of physiological processes, including apoptosis, cell cycle progression, and insulin signaling [25, 26]. Four FOXO members, FoxO1 (FKHR), FoxO3a (FKHRL1), FoxO4 (AFX), and FoxO6, are mostly studied in vertebrates [27–29]. Previous studies indicate that FoxO1 is expressed in a regulated, cell-specific manner during ovarian follicular development, atresia, and luteinization [26, 30, 31]. Recent findings indicate that at the earliest stages of follicular growth, FoxO3a functions as a suppressor of follicular activation and eventually causes signs of premature aging [32]. Therefore, it is important to examine the localization of FoxO3a in the ovaries.

Follicular development, atresia, and luteinization are elaborately regulated by endocrine, paracrine and autocrine factors. Recently, the PKB/Akt pathway has attracted remarkably attention from scientists. Therefore, the aim of the present study was to examine the cellular localization of inhibin α -subunit, PKB/Akt and FoxO3a proteins in the ovaries of Chinese Xiang pigs using immunohistochemistry.

Materials and Methods

Animals and sample collection

Eight Xiang pigs were obtained from the Jiangpu farm of Nanjing Agricultural University, Nanjing, P. R. China. The minipigs used in this experiment had body weights of 20–40 kg and were 5–8 months of age. The animals were kept indoors and were provided with commercial foods three times daily

and tap water *ad libitum*. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

In order to examine the cellular localization of inhibin α -subunit, PKB/Akt and FoxO3a proteins in the ovaries of Chinese Xiang pigs, the ovaries were immediately removed from anesthetized Xiang pigs and fixed in 4% paraformaldehyde at room temperature overnight. After transferring through a graded series of alcohol and xylene, the ovaries were embedded in paraffin and sectioned at 8 μ m thickness. The ovarian sections were mounted on slides and processed for immunohistochemical analysis.

Reagents

The inhibin α -subunit antiserum was obtained from a rabbit immunized against [Tyr30] porcine inhibin α -chain (1–30)-NH₂ conjugated to rabbit serum albumin [33]. This conjugate was kindly provided by Dr. N. Ling (Neuroendocrine Biosciences, La Jolla, CA, USA). Antibodies for FoxO3a (Cat. No. 06-951, Lot 27567) [34] and PKB/Akt (Cat. No. 9472, Lot 1) [35] were obtained from Cell Signaling Technology (Beverly, MA, USA). An ABC Kit was obtained from BioGenex (San Ramon, CA, USA) and 3,3'-diaminobenzidine tetrachloride (DAB) was purchased from Sigma Chemical (St. Louis, MO, USA). All other chemicals were obtained commercially and were of reagent grade.

Immunohistochemistry

Immunohistochemical analysis was conducted in a manner similar to the methods used in our previous reports [10, 25, 36]. Briefly, sections were incubated overnight at room temperature with a polyclonal rabbit immunoaffinity-purified antiserum directed against the inhibin α -subunit (1:2000), FoxO3a (1:100) and PKB proteins (1:100). The specific protein immunoreactivity was visualized with an ABC Kit Elite and 0.05% DAB in 10 mM PBS-buffered saline containing 0.01% H₂O₂ for 5 min. Specificity of the antibody was examined using normal rabbit serum instead of primary antibody. In order to identify structural components and cell types within the ovaries, the sections were counterstained with hematoxylin and mounted with coverslips. Relative levels of immunostaining between animals and cell types

Fig. 1. Cellular localization of inhibin α -subunit during follicular development in the ovaries of Xiang pigs. Positive immunostaining for inhibin α -subunit is indicated by a brown reaction product. Note that inhibin immunoreactivity was detected in the granulosa cells (GCs) of primordial (A), preantral (B), antral (C, D) and antretic follicles (E) and that no significant inhibin staining was detected in theca (TC), stromal and corpora lutea cells (CL, F). In control sections, no significant immunoreactivity was observed when normal rabbit serum was used instead of primary antibody (G, H, I). Bar=50 μ m.

were evaluated by three independent observers, and this was repeated at least four times. The results described herein represent consistently observed patterns of immunohistochemistry.

Results

Localization of inhibin α -subunit

Immunohistochemical analysis of the ovarian tissues revealed that expression of the inhibin α -subunit was found in the granulosa cell (GC) layer of primordial (Fig. 1A), preantral (Fig. 1B), antral (Fig. 1C, 1D), and atretic (Fig. 1E) follicles, but no expression was found in theca cells (TC) and stromal cells. Inhibin immunoreactivity was absent from corpora lutea (Fig. 1F) and was faint in the GCs of primordial follicles. The stained intensity of inhibin immunoreactivity increased in the GCs of preantral and antral follicles and decreased when follicles became atretic. In addition, there was no inhibin immunoreactivity in ovarian vascular endothelial cells (data not shown).

Localization of PKB/Akt

PKB immunoreactivity was found in flat GCs of primordial follicles (Fig. 2A). Interestingly, it was mainly localized in the basal layers of the GCs in antral follicles (Fig. 2B). Immunoreactivity of PKB was also localized in ovarian vascular endothelial cells (data not shown). However, PKB protein was not detected in atretic follicles (Fig. 2C) and corpora lutea (data not shown).

Localization of FoxO3a

FoxO3a immunoreactivity was localized in the ovaries throughout every stage of follicular development, atresia, and luteinization. It was mainly localized in the oocyte cytoplasm and granulosa cells of primary (Fig. 2D), preantral (Fig. 2E), antral (Fig. 2F), and atretic (Fig. 2G) follicles as well as ovarian vascular endothelial cells (data not

shown). In corpora lutea, FoxO3a immunoreactivity was localized specifically in the cytoplasm of luteinized granulosa cells (glc) but not in thecal luteal cells (tlc) (Fig. 2H). No significant localization of FoxO3a was observed in the pycnotic granulosa cells (shown in circles in Fig. 2G) of atretic follicles. These results demonstrate the obligatory function of FoxO3a for the ovaries during the estrous cycle.

Discussion

The findings of the present study clearly demonstrate the cell-specific localization of inhibin α -subunits, PKB, and FoxO3a in the ovaries of Chinese Xiang pigs using immunohistochemical analysis.

Our results from immunohistochemical staining of inhibin α -subunits in the granulosa cells of healthy follicles from Xiang pigs are similar to those obtained in other reports for other animals [10]. However, the results of Kishi *et al.* (2002) demonstrated that inhibin α -subunit is also present in interstitial cells on the first 3 days of the estrous cycle and newly formed luteal cells in the golden hamster [37]. The decrease in stain intensity of the atretic follicles in Xiang pigs is also consistent with observations in other animals [7, 10, 38].

The Akt/PKB signaling pathway is now recognized as one of the most critical pathways regulating cell survival [39–43]. In the present study, it was interesting that PKB was localized specifically at the basal layers of the granulosa cells in antral follicles, which implies that PKB is involved in connection and interaction between theca and granulosa cells. It is well known that synthesis of estradiol is a two-cell process. Whether PKB is involved in this process or not needs further clarification. Ovarian localization of PKB was not limited to follicles, but was also found in blood vascular epithelia. This is in agreement

Fig. 2. Cellular localization of PKB/Akt and FoxO3a during follicular development in the ovaries of Xiang pigs. PKB immunoreactivity was detected in the flat granulosa cells (GCs) of primordial follicles (A) and basal layers of the GCs in antral follicles (B). No significant immunoreactivity of PKB was detected in atretic follicles (C) and corpora lutea (data not shown). FoxO3a immunoreactivity was mainly detected in GCs of primary (D), preantral (E), antral (F), and atretic (G) follicles and was extensive in the cytoplasm of lutealized granulosa cells (glc) but not in the thecal luteal cells (tlc) of corpora lutea (H). No significant localization of FoxO3a was detected in the pycnotic granulosa cells (shown in circles in G) of atretic follicles. In control sections, no significant immunoreactivity was observed when normal rabbit serum was used instead of primary antibody (same as in Fig. 1). Bar=50 μ m.

with the findings of Johnson *et al.* (2001), who showed that Akt plays a more ubiquitous role in the signal pathways of multiple origins [43].

The fact that FoxO3a was localized in follicles and corpora lutea throughout the ovarian cycle in the Chinese Xiang pigs indicates that FoxO3a may play potential roles in the ovary. In addition, there was no PKB immunoreactivity in atretic follicles and corpora lutea when FoxO3a immunoreactivity was localized in the granulosa cells of atretic follicles and in the plasma of granulosa luteal cells in corpora lutea. This indicates that FoxO3a may not only be regulated by PKB, but may also be regulated by other kinases [44, 45].

In conclusion, the fact that cell-specific localization of PKB/Akt and FoxO3a was found in the ovaries of Chinese Xiang pigs indicates that the pathway of PKB and FoxO3a is involved in follicular development, atresia, and luteinization, and some colocalization of inhibin α -subunits,

PKB/Akt, FoxO3a implies that the pathway of PKB/Akt and FoxO3a is involved in the secretion of inhibin, therefore regulating ovarian function in the minipig.

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