Expression and Function of Apoptosis Initiator FOXO3 in Granulosa Cells During Follicular Atresia in Pig Ovaries

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Abstract. In mammalian ovaries, most follicles are lost by atresia before ovulation. It has become apparent that the apoptosis of granulosa cells induces follicular atresia. Forkhead box O3 (FOXO3), also called FKHR (forkhead in rhabdomyosarcoma-like 1), is a proapoptotic molecule that belongs to the FOXO subfamily of forkhead transcription factors. Foxo3-deficient female mice were reported to be infertile because of abnormal ovarian follicular development, but the precise influences of FOXO3 on follicular atresia of mature ovary have not been determined. Therefore, we examined the expression and function of FOXO3 in porcine ovarian follicles and granulosa-derived cells. FOXO3 mRNA levels in granulosa cells of porcine ovaries increased during atresia, while FOXO3 protein was abundant in granulosa cells of early atretic follicles. By immunohistochemistry, the inner surface area of the granulosa layer in early atretic follicles was strongly stained with anti-FOXO3 antibody. The granulosa cells expressing FOXO3 coincided with apoptotic cells, indicating a role of FOXO3 as a proapoptotic factor in granulosa cells of porcine ovaries. In porcine (JC-410) and human (KGN) granulosa-derived cells, cell death was induced by transfection of FOXO3 expression vectors. Expression of the proapoptotic factors Fas ligand (FASLG) and BCL2-like 11 (BCL2L11) was upregulated by FOXO3 in granulosa cells of porcine ovaries. In conclusion, FOXO3 is expressed in porcine ovarian follicles and induces apoptosis in granulosa cells, suggesting that it is a candidate for the initiator of follicular atresia.

Key words: Apoptosis, Forkhead box O3 (FOXO3), Granulosa cell, Porcine ovary

In mammalian ovaries, most follicles degenerate prior to ovulation by a process called atresia [1, 2]. The endocrine regulatory mechanisms involved in follicular development and atresia have been characterized to a large extent [3–5], but the precise temporal and molecular events involved have remained unknown. Studies have suggested that the apoptosis of ovarian granulosa cells plays a major role in follicular atresia [3, 6–8].

The forkhead box O (FOXO) subfamily of forkhead transcription factors, consisting of FOXO1 (FKHR, forkhead in rhabdomyosarcoma), FOXO3 (FOXO3a/FKHRL1, FKHR-like 1), FOXO4 (AFX, acute-lymphocytic-leukaemia-1 fused gene from chromosome X) and FOXO6, participates in diverse processes including cell proliferation, apoptosis, stress resistance, differentiation and metabolism [9, 10]. The transcriptional activity of FOXO is controlled by phosphorylation by a phosphatidylinositol-3-kinase (PI3K)-Akt (PKB, protein kinase B) pathway [10–12]. Three phosphorylation sites have been found in FOXO3: Thr32, Ser253 and Ser315. When phosphorylated, FOXO3 binds to 14-3-3 proteins and is exported out of the nucleus, preventing transcription. Conversely, in the absence of phosphorylation, FOXO3 can translocate to the nucleus and increase the expression of genes.

FOXO3 is known to enhance the transcription of proapoptotic factors such as Fas ligand (FASLG), BCL2-like 11 (BCL2L11, also called Bim) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [10, 13–16], which induces cell apoptosis. The importance of FOXO transcription factors has been demonstrated in mammalian ovaries. Foxo1, Foxo3 and Foxo4 are expressed in granulosa cells of rodent ovaries, and the level of FOXO1 is elevated in granulosa cells of developing follicles [17, 18]. Foxo3-deficient female mice were reported to exhibit infertility due to a global activation of primordial follicles in their ovaries that resulted in subsequent follicular depletion [19, 20], suggesting an essential role of FOXO3 in follicles. However, the expression and role of FOXO3 in granulosa cells of mature ovaries have not been well elucidated.

We first determined the mRNA sequence of porcine FOXO3 and then examined the pattern of FOXO3 expression in porcine ovarian granulosa cells. We then assessed the proapoptotic effect of FOXO3 in porcine ovarian follicles and granulosa-derived cells. FOXO3-deficient female mice were reported to be infertile because of abnormal ovarian follicular development, but the precise influences of FOXO3 on follicular atresia of mature ovary have not been determined.

Materials and Methods

Preparation of porcine granulosa cell samples

As previously reported [23, 24], ovaries were obtained from...
mature sows 100–120 kg in body weight from a local abattoir. Each antral follicle, approximately 2 to 5 mm in diameter, was surgically dissected and opened using fine forceps to obtain the granulosa layer and/or cells under a stereomicroscope. Follicular fluid from each follicle was collected for subsequent progesterone and estradiol-17β analyses. Based on morphological observations of the granulosa cells, each follicle was classified into three types, healthy, early atretic and progressed atretic. Granulosa cells of each follicle type were collected and washed with PBS and then used as samples for extraction of total RNA or protein. Progesterone and estradiol-17β levels of follicular fluid were retrospectively measured using [125I]-RIA kits (BioMerieux, Marcy-l’Etoile, France) to confirm the classification of the follicles. Follicles with a progesterone/estradiol-17β ratio of less than 15 were classified as healthy according to previous findings [23, 25].

Molecular cloning of porcine FOXO3

The full-length porcine FOXO3 sequence was determined by 5′- and 3′-rapid amplification of cDNA ends (RACE). Total RNA was prepared from porcine granulosa cells using an RNeasy Mini kit (Qiagen, Chatsworth, CA, USA). Then, 5′-RACE and 3′-RACE were performed using a Genecatcher kit (Invitrogen, Carlsbad, CA, USA) as described previously [26]. The primers for determining the 5′- and 3′-ends of porcine FOXO3 were designed from a partial sequence of Sus scrofa FOXO3 in the GenBank database. Amplified 5′- and 3′-cDNA fragments were ligated into the vector pCR4-TOPO (Invitrogen) and then subjected to sequence analyses. Nucleotide sequences of FOXO3 were analyzed with the Genetyx software (Software Development, Tokyo, Japan).

RNA extraction, cDNA synthesis, and real-time RT-PCR

Porcine tissues were obtained from mature sows at a local abattoir. Total RNA was extracted from granulosa cells or tissues with an RNeasy mini kit (Qiagen), and first strand cDNA was synthesized from total RNA using a Ready-To-Go T-Primed First-Strand kit (Sigma Aldrich Chemicals, St. Louis, MO, USA) and 1% (w/v) Dithiothreitol (DTT; Wako) to confirm the classification of the follicles. Follicles with a progesterone/estradiol-17β ratio of less than 15 were classified as healthy according to previous findings [23, 25].

Immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining

The ovaries obtained from mature sows were fixed in 20% (v/v) buffered formalin (pH 7.4) and embedded in paraffin (Histosec; Merck, Darmstadt, Germany). Serial sections were cut and used for immunohistochemistry and TUNEL staining [28–30]. For immunohistochemistry, ovarian sections were deparaffinized, and immersed in methanol with 0.3% (v/v) H2O2. Normal goat serum (Sigma) was placed on each section, and then the sections were incubated with rabbit anti-human FOXO3 antibody (Upstate) diluted 1:250 with PBS containing 1% (w/v) BSA (Sigma) for 1 h at RT. Subsequently, the sections were incubated with biotinylated anti-rabbit IgG (1:200; Dako, Glostrup, Denmark) for 1 h at room temperature (RT; 23–25 °C). The immune complexes were detected using an ECL Western blot detection reagent hydrogen peroxide kit (Amersham Pharmacia Biotech) and recorded with a digital fluorescence recorder (LAS-1000; Fujifilm, Tokyo, Japan).

Protein extraction and Western blotting

Proteins of granulosa cells and cultured cells were extracted as previously described [27]. In brief, cultured cells were washed with PBS (pH 7.4), treated with 10% trichloroacetic acid (TCA; Wako Pure Chemical, Osaka, Japan), scraped from culture plates and lysed in UTD buffer [9 M Urea (Wako), 2% (v/v) Triton X-100 (Sigma Aldrich Chemicals, St. Louis, MO, USA) and 1% (w/v) (±)-Dithiothreitol (DTT; Wako)]. After treatment with an ultrasonic cell disruptor, 2% (w/v) lithium dodecyl sulfate (Wako) and 0.03 M Tris (Trizma Base; Sigma) were added. As previously described [28], 5 µg of protein in each sample was fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF membrane; Millipore, Bedford, MA, USA). After incubation in blocking buffer [10 m M Tris-HCl, pH 8.0, 100 m M NaCl, 0.1% (v/v) Tween 20 (Sigma) and 2.5% (w/v) bovine serum albumin (BSA; Sigma)], the membranes were incubated with anti-human FOXO3 antibody (diluted with blocking buffer at 1:600; Upstate, Lake Placid, NY, USA), anti-phospho-FOXO3 (Thr25) (1:600; Upstate), anti-phospho-FOXO3 (Ser25) (1:600; Upstate), anti-human FASLG antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-human BCL2L11 antibody (1:200; Calbiochem, La Jolla, CA, USA) or anti-human GAPDH antibody (1:250; Santa Cruz Biotechnology) at 4°C for 18 h and then washed in washing buffer (10 m M Tris-HCl, pH 7.5, 100 m M NaCl and 0.1% (v/v) Tween 20). Then, the membranes were treated with secondary antibody [horseradish peroxidase (HRP)-conjugated IgG, diluted with washing buffer at 1:2,000; Dako, Glostrup, Den mark] for 1 h at room temperature (RT; 23–25 °C). The immune complexes were detected using an ECL Western blot detection reagent hydrogen peroxide kit (Amersham Pharmacia Biotech) and recorded with a digital fluorescence recorder (LAS-1000; Fujifilm, Tokyo, Japan).

Cell culture

The human ovarian granulosa-like tumor cell line, KGN, and
stable porcine granulosa cell line, JC-410, were used in the experiments. These cells were cultured at 37°C with 5% CO₂. KGN cells were cultured in DMEM/F12 (Invitrogen) with 10% fetal bovine serum (Sigma). Medium 199 (Invitrogen) with 5% newborn calf serum (Invitrogen) and 4 μg/ml of porcine recombinant insulin (Sigma) was used for culturing JC-410 cells. All the media contained 100 μg/ml penicillin and 100 μg/ml streptomycin.

**Transient transfection**

Human FOXO3 expression vectors, pECE-FOXO3a-Wildtype (FOXO3-WT) and pECE-FOXO3a-Triple mutant (FOXO3-TM), were provided by Dr. Michael Greenberg (Harvard Medical School, Boston, MA, USA) [10]. These expression vectors or empty vector were transiently transfected into KGN or JC-410 cells as described previously [27]. Cells were plated 24 h before transfection, and then the vectors were introduced with Lipofectamine 2000 (Invitrogen). At 24 or 48 h after transfection, cell viability was determined by MTS assay or protein was extracted from the cells as described above.

**Cell viability assay**

The MTS assay was performed to determine cell viability using CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI, USA) following the manufacturer’s instructions. Thirty micro-liters of CellTiter 96 AQueous One Solution Reagent was added to each well of 96-well assay plates containing 150 μl of culture medium. The plates were incubated for 2 h at 37°C in a CO₂ incubator (5% CO₂ in the air), and then the absorbance at 490 nm was measured using a 96-well plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

**Statistical analysis**

The Student’s t-test was performed using the StatView IV program (Abacus Concepts, Berkeley, CA, USA). Each value represents the mean ± SEM. Differences of P<0.05 were considered significant.

**Results**

**Molecular cloning of porcine FOXO3**

Total RNA was obtained from porcine granulosa cells, and the full-length mRNA sequence of pig FOXO3 was determined using 5′- and 3′-RACE methods. The mRNA sequence was then deposited in the GenBank database (accession number AB433530, date of accession; 9th Apr 2008). The sequence of porcine FOXO3 was highly conserved, being 92.0% homologous with human FOXO3 and 90.7% homologous with murine Foxo3 at the mRNA level. The homology of the estimated amino acid sequence was higher, 96.3 and 94.3% with the human and mouse sequence, respectively. Importantly, the sequences of the DNA-binding domain and three phosphorylation sites (Thr²⁵, Ser²⁵³ and Ser³¹⁵) were completely conserved among pigs, humans and mice at the amino acid level (Fig. 1).

**Levels of FOXO3 mRNA in various tissues**

The levels of FOXO3 mRNA in various porcine tissues (granulosa cells of healthy follicles, cerebrum, cerebellum, medulla oblongata, salivary gland, thymus, lung, heart, liver, spleen, stomach, small intestine, large intestine, kidney, adrenal gland and muscle) were examined by real-time RT-PCR (Table 1). FOXO3 mRNA was expressed in all tissues examined. The expression was particularly strong in the stomach and large intestine. In the heart and muscle, the expression was relatively weak.

**Levels of FOXO3 mRNA in porcine granulosa cells**

The levels of FOXO3 mRNA in granulosa cells at different stages (healthy, early atretic and progressed atretic follicles) were analyzed by real-time RT-PCR, and the levels normalized to the expression of GAPDH are shown in Fig. 2. Lower levels of FOXO3 mRNA were demonstrated in granulosa cells of healthy follicles, and the expression increased significantly in those of early and progressed atretic follicles.

**Expression of FOXO3 protein in porcine granulosa cells**

FOXO3 protein in porcine granulosa cells was detected by Western blotting (Fig. 3). Representative photographs are shown in Fig. 3A, and the expression normalized to that of GAPDH is shown in Fig. 3B. FOXO3 protein, 95 kDa, was detected in granulosa cells at all stages, but the level became significantly higher in granulosa cells of early atretic follicles and was lowest in those of progressed atretic follicles.

**Localization of FOXO3 protein in pig ovaries**

The localization of FOXO3 protein in porcine ovaries was visualized by immunohistochemical staining (Fig. 4). Strong immunostaining was observed in granulosa cells located on the inner surface of the follicular wall in early atretic follicles (Fig. 4C). In contrast, immunohistochemical staining was relatively weak in granulosa cells of healthy and progressed atretic follicles (Fig. 4A and E, respectively). The stages of follicular development were certified by TUNEL as follows: no signal in the granulosa layer in healthy follicles (Fig. 4B), staining of the inner cells of the granulosa layer in early atretic follicles (Fig. 4D) and strong staining of the entire granulosa layer in progressed atretic follicles (Fig. 4F).

**Induction of cell death by FOXO3 in granulosa-derived cell lines**

FOXO3 expression vectors were transiently transfected into KGN and JC-410, and cell viability was examined by MTS assay. Cell death was induced by FOXO3-WT and FOXO3-TM both in human (KGN) and pig (JC-410) granulosa cell lines (Fig. 5A and B, respectively). The excess expression of FOXO3 protein in the transfected KGN cells was confirmed by Western blotting (Fig. 5C). FOXO3 antibody detected increased amounts of the protein in cells transfected with FOXO3-WT and FOXO3-TM vectors. By contrast, anti-phospho-FOXO3 antibodies (Thr²⁵ and Ser²⁵³) detected excess expression of phosphorylated FOXO3 only in cells transfected with FOXO3-WT, as the FOXO3-TM vector is mutated to avoid phosphorylation.
<table>
<thead>
<tr>
<th></th>
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<th>mFOXO3</th>
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Fig. 1. Estimated amino acid sequences of pig, human and mice FOXO3 (pFOXO3, hFOXO3 and mFOXO3, respectively). Asterisks indicate the residues differ among species. The DNA binding domain and three phosphorylation sites are highlighted with grey and black shading, respectively, and are completely conserved among the three species.
Effect of FOXO3 overexpression on protein expression of FASLG and BCL2L11

To examine if FASLG and BCL2L11 are the transcriptional targets of FOXO3 in granulosa cells, the levels of these protein in KGN cells transfected with FOXO3 were determined by Western blotting (Fig. 6). Both FOXO3-WT and FOXO3-TM vectors induced higher levels of production of FASLG protein than the control vector. The level of BCL2L11 was also increased in KGN cells by the overexpression of FOXO3.

Discussion

Our sequence analyses showed that porcine FOXO3 mRNA and amino acid sequences were highly conserved among pigs (NCBI Reference Sequence accession number NM_001135959.1), humans (NM_001455.3) and mice (NM_019740.2). This strengthens the common importance of FOXO3 in many mammalian species. In addition, FOXO3 was expressed in a broad range of porcine tissues in the present study, indicating that it is essential for many tissues. While FOXO3 is widely expressed, the most notable dysfunction appeared in ovaries by Foxo3 gene knockout [19, 20], suggesting its key role in ovarian function.

Foxo3-null female mice exhibit abnormal ovarian follicular development, which results from excessive activation from primordial to primary follicles that occurs soon after birth [19, 20, 31]. In ovaries of postnatal mice and rats, Foxo3 is mostly expressed in oocytes of primordial and primary follicles, rather than in granulosa cells [32]. In granulosa cells of mature rodents, the quantity of Foxo3 mRNA expression does not differ among follicular developmental stages, but the localization and expression level of FOXO3 protein have not been determined [17]. Our immunohistochemical

Table 1. Expression levels of FOXO3 mRNA in porcine tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>FOXO3/GAPDH</th>
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<tbody>
<tr>
<td>Granulosa cell (healthy follicle)</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>2.0±0.7</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>4.3±0.7</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>19.1±4.3</td>
</tr>
<tr>
<td>Thymus</td>
<td>21.7±2.9</td>
</tr>
<tr>
<td>Lung</td>
<td>11.7±1.1</td>
</tr>
<tr>
<td>Heart</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>7.4±1.2</td>
</tr>
<tr>
<td>Spleen</td>
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</tr>
<tr>
<td>Stomach</td>
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</tr>
<tr>
<td>Small intestine</td>
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<tr>
<td>Large intestine</td>
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</tr>
<tr>
<td>Kidney</td>
<td>2.5±0.3</td>
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<tr>
<td>Adrenal gland</td>
<td>8.71±2.1</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.2±0.0</td>
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</table>

The levels of FOXO3 mRNA in various porcine tissues were examined by real-time RT-PCR. Measurement values of FOXO3 were normalized to the GAPDH level and are presented as fold expression of granulosa cells of healthy follicles. Means ± SEM from three independent experiments are shown.

Fig. 2. The levels of FOXO3 mRNA in porcine granulosa cells of healthy (H), early atretic (EA) and progressed atretic (PA) follicles were examined by real-time RT-PCR. As an intrinsic control, GAPDH mRNA expression was measured in the same samples. Measurement values of FOXO3 were normalized to the GAPDH level and are presented as fold expression of granulosa cells of healthy follicles. Means ± SEM from three independent experiments are shown. Asterisks indicate significant difference (*: P<0.05).

Fig. 3. The levels of FOXO3 protein in porcine granulosa cells of healthy (H), early atretic (EA) and progressed atretic (PA) follicles were examined by Western blotting. Representative photographs are shown in A, together with GAPDH levels as an internal control. FOXO3 protein levels were normalized to the GAPDH level and are represented as fold expression of granulosa cells of healthy follicles (B). Means ± SEM from three independent experiments are shown. Asterisks indicate significant difference (**: P<0.01).
Fig. 4. Pig ovarian sections were stained immunohistochemically with a FOXO3 antibody (A, C and E), and apoptotic cells were detected in serial sections by TUNEL staining (B, D and F). Representative photographs of a healthy follicle (A and B), early atretic follicle (C and D) and progressed atretic follicle (E and F) are shown. An arrowhead indicates positive staining for FOXO3 in the granulosa cell layer. g: granulosa cell layer. ti: theca interna layer. Original magnification ×400.

Fig. 5. Human FOXO3 expression vectors were transfected into cultured cells, and cell viability was determined (A and B). Empty vector (Vector), FOXO3-WT vector or FOXO3-TM vector was introduced into KGN (A) or JC-410 (B) cells, and an MTS assay was performed 24 h (grey bar) and 48 h (black bar) after transfection. Means ± SEM, presented as % of Vector, from six independent experiments are shown. Asterisks indicate significant difference vs. Vector (* and **: P<0.05 and 0.01, respectively). The excess expression derived from the FOXO3 expression vectors in KGN cells was confirmed by Western blotting using antibodies against FOXO3 and phospho-FOXO3 (FOXO3-Thr32, FOXO3-Ser253) (C). Proteins of KGN cells were obtained 36 h after transfection. The level of GAPDH protein was determined as an intrinsic control.
experiment on mature porcine ovaries indicated FOXO3 staining in the granulosa layer, but only very weak staining in oocytes (data not shown). Meng et al. also reported the localization of FOXO3 protein in granulosa cells of mature pig ovaries [33]. In ovaries of mature pigs, FOXO3 might have critical roles in granulosa cells rather than oocytes.

The expression of FOXO3 mRNA in porcine ovarian granulosa cells was increased during atresia, while its protein level was highest in granulosa cells of early atretic follicles; this might be because protein degradation is promoted or the ability to translate the mRNA into protein had been lost in granulosa cells of progressed atretic follicles. The region stained by FOXO3 antibody closely coincided with that stained by the TUNEL method, showing that FOXO3 protein is expressed in the inner granulosa layer in the very early phase of atresia. Western blotting and immunohistochemistry with anti-phospho-FOXO3 antibodies detected trace levels of phosphorylated FOXO3 (Thr32 and Ser253) in porcine ovarian granulosa cells (data not shown).

When KGN and JC-410 cells were transfected with FOXO3, their viability decreased significantly, showing that FOXO3 acts as an anti-survival factor of granulosa cells. The effects of the FOXO3-WT and FOXO3-TM vectors did not differ significantly in KGN or JC-410 cells, implying that many of the FOXO3 proteins are activated (unphosphorylated) in granulosa cells after transfection. Considering this with the trace expression of phospho-FOXO3 in porcine ovarian granulosa cells, the amount of FOXO3 should be important in granulosa cells, rather than its phosphorylation.

In KGN cells, the levels of FASLG and BCL2L11 were increased by FOXO3. As such, FOXO3 may induce cell death by enhancing expressions of those apoptotic factors. FASLG is one of the main apoptosis-inducible factors in granulosa cells of many mammalian species [34–36]. On the other hand, the contribution of BCL2L11 to granulosa cell apoptosis has not been reported yet. It is possible that BCL2L11 is also involved in granulosa cell apoptosis via transactivation by FOXO3. We tried to examine levels of FASLG and BCL2L11 in JC-410 cells, but it was difficult to obtain a sufficient amount of JC-410 protein after gene transfection. The amount of TRAIL, which was reported to induce apoptosis in pig granulosa cells [24], was not changed in the same KGN sample (data not shown).

Up to now, the PI3K-Akt-FOXO3 pathway has been indicated to have a central role in the mammalian oocyte during primordial follicle activation and early follicular development [37, 38]. Here, we demonstrated another function of ovarian FOXO3, a proapoptotic role in granulosa cells of the mature ovary. We indicated the importance of the amount of FOXO3 in granulosa cell apoptosis, but the factor(s) that upregulates FOXO3 remains to be examined in the future.

In conclusion, FOXO3 is expressed in porcine ovarian follicles, especially in granulosa cells of early atretic follicles, and induces apoptosis in granulosa cell lines, suggesting that it is a candidate for the initiator of follicular atresia.

Acknowledgments

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