Monoclonal Antibodies Recognize a Novel Cell Death Receptor and a Decoy Receptor on Granulosa Cells of Porcine Ovarian Follicles

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Abstract: *We prepared IgM and IgG (PFG-5 and PFG-6, respectively) monoclonal antibodies against granulosa cells prepared from healthy antral follicles of porcine ovaries. PFG-5 antibody specifically recognized two cell-membrane proteins (PFG-5 antigen: 55 kD, pI 5.9, and PFG-6 antigen: 42 kD, pI 5.2), and PFG-6 antibody recognized PFG-6 antigen. Immunochemical reactions of these antibodies were only detected in follicular granulosa cells but not any other ovarian tissues or organs. Both antigens were detected in granulose cells of healthy follicles, but PFG-6 antigen disappeared in granulosa cells of atretic follicles. When the isolated granulosa cells prepared from healthy follicles were cultured in medium containing PFG-5 antibody, the cells underwent apoptosis, and co-incubation with PFG-6 antibody inhibited PFG-5 antibody inducible apoptosis. These observations suggested that PFG-5 antigen is a novel cell death receptor, which is different from well-known apoptosis-mediating receptors (Fas or tumor necrosis factor receptor), and that PFG-6 antigen may act as a decoy receptor and inhibit apoptotic signals through PFG-5 antigen.*

Key words: *Apoptosis, Cell death receptor, Decoy receptor, Granulosa cell, Porcine ovary*

In mammalian ovaries, more than 99.9% of the follicles undergo the degenerative change known as atresia at varying stages of follicle development [1, 2]. A number of studies of follicular atresia have revealed the morphological and biochemical characteristics of atretic follicles [3–6]. Recent findings have suggested that apoptosis, originally described by Kerr *et al.* [7], is the mechanism underlying ovarian follicular atresia. Apoptotic cell death of granulosa cells of rabbit Graafian follicles with atresia was first observed by Flemming [8], who called it "chromatolysis". Unfortunately, the physiological roles of chromatolysis in granulosa cell of atretic follicles are not well understood. The degeneration of atretic follicles in mammalian ovaries can be explained, at least in part, by apoptotic cell death of granulosa and theca interna cells [3–6, 9]. However, the degenerative changes in cumulus cells during follicular atresia have not been investigated in detail [6]. Recently, we confirmed that apoptosis occurs in granulosa cells but not cumulus cells in the atretic Graafian follicles from porcine ovaries [10–24]. Briefly, *in situ* analysis of DNA fragmentation was performed on histological sections of follicles using the TUNEL method, and then conventional electron microscopic analysis was also performed. In healthy follicles, no apoptotic cells were observed among granulosa or cumulus cells, internal or external theca cells, or oocytes. In the early stage of atresia, apoptosis demonstrated histochemically by TUNEL staining was seen in scattered granulosa cells located on the inner surface of the follicular wall, but not in cumulus cells, internal or external theca cells, or oocytes [10–18]. Typical apoptotic features, i.e. condensed nuclei, were seen in the scattered granulosa cells, but cumulus cells with normal ultrastructure were also seen in the same follicles [21]. In the late to final stages of atresia, granulosa cells scattered on the inner surface of the follicular wall began to undergo apoptosis, but no TUNEL-positive cells were detected among the cumulus cells. Moreover, the neutral Ca^{2+}/Mg^{2+}-dependent endonuclease, and not the neutral Ca^{2+}-dependent endonuclease, neutral Mg^{2+}-dependent endonuclease or acidic cation-independent endonuclease, is involved in

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granulosa cell apoptosis of the atretic antral follicles [13]. No endonuclease activity was detected in cumulus cells prepared from the same atretic follicles. These histological, cytological and biochemical findings confirmed that there were no apoptotic changes in the cumulus cells of the atretic follicles in immature or mature porcine ovaries [10–26].

Previously, we prepared an IgM monoclonal antibody (named PFG-1) capable of inducing granulosa cell apoptosis [27, 28]. PFG-1 was produced against granulosa cells prepared from healthy antral follicles of porcine ovaries, and specifically recognized a cell membrane glycoprotein (named PFG-1 antigen) with a molecular weight of 55 kD and isoelectric point of 5.9. When the isolated granulosa cells prepared from healthy follicles were cultured in medium containing PFG-1, the cells underwent apoptosis as determined by nuclear morphology, DNA electrophoresis and flow cytometric analysis [27, 28]. Immunochemical and biochemical characteristics of the PFG-1 antigen were different from those of known apoptosis-mediating receptors, Fas/Apo-1/CD95 or tumor necrosis factor (TNF) receptors, suggesting that the PFG-1 antigen is a new cell death receptor located on the granulosa cells of antral follicles in porcine ovaries. However, mechanisms of regulation of this novel cell death receptor are not well understood [27, 28]. In the present study, we produced two unique monoclonal antibodies to reveal the molecular mechanisms regulating granulosa cell apoptosis in porcine follicles. An IgM monoclonal antibody (PFG-5), which is capable of inducing granulosa cell apoptosis, and an IgG monoclonal antibody (PFG-6), which is not capable of inducing granulosa cell apoptosis and inhibits the PFG-5-induced apoptosis, have been produced against granulosa cells prepared from healthy antral follicles.

Materials and Methods

Preparation of follicular granulosa cells

Granulosa cells from healthy antral follicles of porcine ovaries were prepared as described previously [27, 28]. Briefly, ovaries were obtained from mature pigs at a slaughterhouse, and then individual preovulatory antral follicles, 4–5 mm in diameter, were dissected in Medium 199 (Gibco BRL, Grand Island, NY, USA) with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; Sigma Aldrich Chemicals, St. Louis, MO, USA) and 0.1% polyvinylalcohol (Wako Pure Chemical, Osaka, Japan) from the ovaries. Under a surgical dissecting microscope (SZ11; Olympus, Tokyo, Japan), follicles were classified as morphologically healthy or atretic [29] (Fig. 1A). The healthy follicles were punctured over 1.5-ml microcentrifuge tubes to collect follicular fluid. Fluid from each follicle was separated by centrifugation, and then estradiol-17β and progesterone levels were measured by [^{125}I]-radioimmunoassay (RIA) as described below to confirm the classification of the follicles [10–17]. Then, the granulosa cell layers were removed from the follicles in 25 mM HEPES-buffered Medium 199 containing 80 mg/ml kanamycin sulfate (Sigma) (HEPES-199) (Fig. 1B). After washing with HEPES-199, the cell layers were incubated in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (Gibco) containing 10 mM ethylenediaminetetraacetic acid disodium salt (EDTA; Gibco) and 6.8 mM ethyleneglycol-bis-tetraacetic acid (EGTA; Sigma) for 15 min at room temperature (RT; 22–25°C), and then granulose cells were isolated by pipetting. The isolated cells were washed twice in HEPES-199 containing 10% fetal calf serum (FCS; Gibco) (HEPES-199-FCS) by centrifugation. Cell number was counted using a hemocytometer plate, and cell viability was determined by the trypan blue exclusion method. The isolated cells with viability of more than 95% were used as antigens for immunization and as target cells in cell-killing activity assay as described below.

RIA of steroid hormones in the follicular fluid

Estradiol-17β and progesterone levels in follicular fluid diluted 100-fold with Medium 199 were quantified using [^{125}I]-RIA kits (Bio-Mérieux, Marcy-l'Etolle, France) as described previously [10–17]. In pigs, the progesterone/estradiol-17β ratio of follicular fluid in each follicle provides a good index of follicular atresia [30–32]. When the progesterone/estradiol-17β ratio of follicular fluid was less than 15, the follicle was classified as healthy according to our previous findings [10–17].

Preparation of monoclonal antibodies

Eight-week-old female BALB/c mice purchased from Clea Japan (Tokyo, Japan) were used. All animals received humane care as outlined in the "Guide for the Care and Use of Laboratory Animals" (Kyoto University Animal Care Committee according to NIH #86-23; revised 1999). They were housed in a controlled environment (lights on between 7:00 and 19:00; temperature 22 ± 2°C; humidity 70 ± 5%) and were immunized intravenously with isolated healthy granulosa cells (10^6 cells/mouse, biweekly). Immunization was repeated four to six times. Antibody production was assessed by conventional immunofluorescence staining as described below. Five days after the last

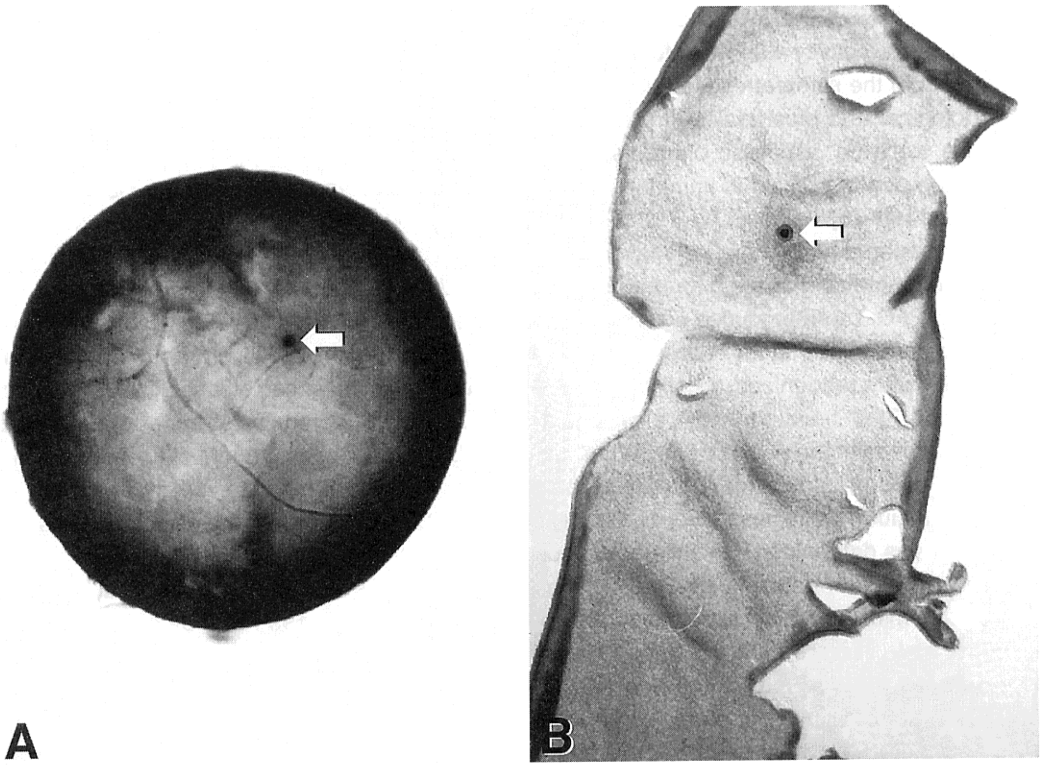

Fig. 1. Healthy follicle, 4.6 mm in diameter, dissected from porcine ovary under a surgical dissecting microscope (A). Granulosa cell layer removed from the healthy follicle (B). Oocyte (arrow in A) was seen from outside of healthy follicle (A), and the oocyte-cumulus cell complex (arrow in B) tightly contacted with granulosa cell layer in healthy follicle (B). × 40 and 60.

immunization, fewer cells (10^4 cells/mouse) were injected intravenously as a booster injection. Three days after boosting, the spleen cells from immunized mice, which produced anti-granulosa cell antibodies, were fused with Sp2/O-Ag14 mouse myeloma cells by standard hybridization techniques using polyethylene glycol 1500 (Boehringer Mannheim, Indianapolis, IN, USA) [33]. After washing with Iscove's modified Dulbecco's medium (IMDM; Gibco), the fused cells were suspended in IMDM containing 10% FCS and 1 unit/ml of interleukin 6 (IL6; Sigma) (IMDM-FCS-IL6), and plated in 96-well cell culture plates (Falcon 3872; Becton Dickinson, Lincoln Park, NJ, USA). After 24 h incubation at 37°C in 5% CO_2, hypoxanthine-aminopterin-thymidine (HAT) medium (1×10^{-4} M hypoxanthine, 0.8×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine; Boehringer) was added to each well. Every 3 days, half of the culture medium was removed and replaced with IMDM-FCS-IL6 containing HAT medium (IMDM-FCS-IL6-HAT). After three changes of IMDM-FCS-IL6-HAT, the hybridoma cells were incubated in IMDM-FCS-IL6 containing hypoxanthine-thymidine (HT) medium (1×10^{-4} M hypoxanthine and 1.6×10^{-5} M thymidine; Boehringer) (IMDM-FCS-IL6-HT). Thereafter, half of the culture supernatant was replaced with fresh IMDM-FCS-IL6-HT every 3 days. The hybridoma cells producing antibodies against the granulosa cell surface were screened by conventional immunofluorescence staining. Then, antibody class was determined by ELISA as described previously [27, 28]. Two hybridoma cell lines, which produced IgM and IgG antibodies against the granulosa cell surface, were selected, cloned twice by limiting dilution, and named PFG-5 and PFG-6, respectively.

Four-week-old female BALB/c mice received intraperitoneal injection of 0.5 ml/mouse of pristane (2, 6, 10, 14-tetramethylpentadecane; Sigma). One month after pristane injection, PFG-5 and PFG-6 hybridoma cells (1×10^7 cells/mouse) were injected intraperitoneally. Within two weeks after injection, ascites were obtained from the mice and dialyzed against PBS (pH 7.4), and then immunoglobulin-rich fractions were precipitated with 50% saturated ammonium sulfate (Wako). These crude antibodies were applied to a hydroxyapatite column (Asahi Optical Inc., Tokyo, Japan). The IgM and IgG fractions were eluted with a 10–400 mM gradient of sodium phosphate, pH 7.4, by preparative HPLC (Pharmacia Biotech, Uppsala, Sweden). Eluted antibodies concentrated in ultrafiltration cells with XM50 ultrafiltration membrane (Amicon, Beverly, MA, USA) were heat-inactivated for 45 min at 56°C and sterilized by filtration through 0.22-μm porefilters (Millipore, Marlborough, MA, USA). The optical densities at 280 nm of the antibody solutions were measured with a spectrophotometer (Ultrospec 3000; Pharmacia) to determine protein concentration.

Immunofluorescence staining

Porcine ovaries obtained at a slaughterhouse were cut into small pieces, put on filter paper, mounted in OCT compound (Miles Lab., Elkhart, IN, USA), and then rapidly frozen in liquid nitrogen. Serial sections (5 μm thick) were cut on a cryostat (Jung CM1500; Leica, Heidelberger, Germany), mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Sigma), and fixed with precooled acetone for 5 min at −80°C. After washing with PBS, the sections were preincubated with 1% normal goat serum (Sigma) diluted with PBS containing 1% bovine serum albumin (BSA; Sigma) (PBS-BSA) for 2 h at RT. The slides were washed with PBS containing 0.05% Tween 20 (Sigma) (PBS-Tw), and then the sections were incubated with mouse serum (1/10 dilution with PBS-BSA), hybridoma culture supernatant (1/100 dilution with PBS-BSA) or purified monoclonal antibody (1/400 dilution with PBS-BSA) for 18 h at 4°C. As negative controls, adjoining sections were incubated with diluted normal mouse serum (1/10 dilution with PBS-BSA) or mouse IgM or IgG (1 μg/ml in PBS-BSA; Sigma). After washing with PBS-Tw, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM or IgG antibody (1/400 dilution with PBS-BSA; American Qualex, La Mirada, CA, USA) for 2 h at RT. After washing with PBS-Tw, the sections were examined with a fluorescence microscope (BX50-fluoro system, Olympus) or a confocal laser scanning microscope (Fluoroview FV3000, Olympus).

Western blotting analyses

For conventional Western blotting analysis, homogenized samples of ovarian tissues (granulosa cells and luteal bodies), oviduct, uterus, testis, liver, kidney, adrenal gland, pancreas, stomach, small intestine, large intestine, spleen, thymus, brain, heart, lung or skeletal muscle were electrophoresed through sodium dodecyl sulfate (SDS)-4% polyacrylamide slab gels as described previously [27, 28]. After SDS-polyacrylamide gel electrophoresis (PAGE), separated protein bands in the gels were stained with a Coomassie brilliant blue staining kit (Wako) according to the manufacturer's instructions.

As previously reported [27, 28], cell membrane samples of the isolated granulosa cells were separated by two-dimensional PAGE (2D-PAGE) which was performed according to the method of O'Farrell [34]. Briefly, the cell membrane samples prepared from healthy and atretic follicles were solubilized in 2% Triton X-100 (Sigma) containing 6 M urea, 5% 2-mercaptoethanol and 2% carrier ampholyte (pH 3.5–10; Pharmacia). The lysates were separated by isoelectric focusing on cylindrical 4% polyacrylamide gels, separated by SDS-PAGE using 7.5% polyacrylamide slab gels, and then separated protein spots in the gels were stained with a Coomassie brilliant blue staining kit.

After SDS-PAGE or 2D-PAGE, the proteins were transferred onto nitrocellulose membranes (Wako), which then were preincubated with 3% (w/v) skimmed milk in PBS for 1 hr at 37°C. After washing with PBS-Tw, the membranes were incubated with monoclonal antibodies at 10 μg/ml, and immunological reaction products were visualized with an ABC staining kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions.

Cell-killing activity assay

Cell-killing activity was determined by a apoptotic cell determination kit (Wako) according to the manufacturer's instructions. Briefly, the isolated healthy granulosa cells (10^6 cells/ml) in 96-well culture plates were cultured in HEPES-199-FCS containing 10% each hybridoma culture supernatant or monoclonal antibody (0.0001 to 1,000 μg/ml of PFG-5 and/or PFG-6) for 3 to 72 h at 37°C. As a negative control, the granulosa cells were cultured in HEPES-199-FCS with mouse immunoglobulin or without any additives. The cells were resuspended by pipetting and incubated with detective medium, then viable cells were quantified by a fluorescence microscope.

Histochemistry for determination of apoptosis

Cultured granulose cells were stained by the terminal deoxynucleotidyl transferase-mediated biotinylated deoxyuridine triphosphate nick end-labeling (TUNEL) method using a commercial kit (Apop Tag; Oncor Inc., Gaithersburg, MD, USA) as described previously [10–18] to determine the apoptotic cells. The nuclei of cultured cells were stained with Hoechst 33258 (Molecular Probes, Eugene, OR, USA) to observe their morphology. Briefly, the isolated healthy granulosa cells

(10^6 cells/ml) on 13-mm plastic coverslips (Nunc 174650, Intermed, Tokyo, Japan) placed in the wells of 24-well culture plates (Falcon 3047, Becton Dickinson) were cultured in HEPES-199-FCS containing monoclonal antibody (0.0001 to 1,000 μg/ml of PFG-5 and/or PFG-6) for 3 to 72 h at 37°C. As a negative control, the cells were cultured in HEPES-199-FCS with mouse immunoglobulin or without any additives. For TUNEL staining, the cultured cells attached to the plastic coverslips were fixed in 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan) in 0.1 M phosphate buffer (pH 7.4) for 10 min at 4°C, then incubated with proteinase K (10 μg/ml; Sigma) for 10 min at 20°C, and washed in distilled water. The cells were incubated with terminal deoxynucleotidyl transferase (TDT; 1 U/μl; Boehringer) solution containing 45 μM ddATP and 5 μM digoxigenin-11-2',3'-deoxyuridine-5'-triphosphate (DIG-ddUTP; Boehringer) for 1 h at 37°C, and then immersed in double-strength salt sodium citrate buffer. After washing with PBS, the cells were incubated with FITC-labeled anti-DIG antibody solution (Boehringer) at 1:100 dilution with PBS containing 2% BSA for 1 h at RT. After washing with PBS, the cells were examined with a fluorescence microscope or a confocal laser scanning microscope. The following positive and negative controls were included in each experimental run: as negative controls, the cell samples were incubated with omission of either TDT or DIG-ddUTP; as a positive control, the cell samples were treated with DNase I (1 μg/ml; Boehringer) for 5 min at RT before exposure to TDT, and paraffin sections prepared from young adult rat testis were used as physiological positive controls [11]. To observe the morphology of the nuclei, the cultured cells attached to the plastic coverslips were fixed with cold acetone (−80°C) for 5 min, washed with PBS, and stained with Hoechst 33258 (4 μg/ml in distilled water) for 5 min. After washing with PBS, the cells were examined with a fluorescence microscope.

DNA electrophoresis

To assess the DNA fragmentation in cultured granulosa cells, DNA samples prepared from cultured cells were electrophoresed [27, 28]. Briefly, after incubation with monoclonal antibody as described above, cultured granulosa cells were resuspended by pipetting, and washed with modified HEPES-199-FCS. DNA fractions were separated from the cells by centrifugation at 9,000 g for 20 min at 4°C. DNA contents were determined by 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma)-fluorescence assay using calf thymus DNA as a standard [35], and then DNA samples were electrophoresed in 2% agarose gels with 40 mM Tris-acetate buffer, pH 8.1, containing 2 mM EDTA, 18 mM NaCl, and 10 μg/ml ethidium bromide at 60 V for 90 min. Gels were photographed on an ultraviolet transilluminator.

Results

Immunohistochemical characterization of monoclonal antibodies

Two hybridoma cell lines, named PFG-5 and PFG-6, producing IgM and IgG antibodies to porcine granulosa cell-surface components, were selected and cloned. The characteristics of the monoclonal antibodies produced by these hybridoma cell clones were immunohistochemically determined. Immunofluorescence staining on serial cryostat sections of porcine ovaries was used to determine the target specificity of the monoclonal antibodies. PFG-5 showed strong fluorescent staining on granulosa cells of healthy (Fig. 2) and atretic follicles. PFG-6 antibody was reactive with granulosa cells of healthy follicles. These antibodies did not label theca interna or externa cells, basement membrane, or ovarian stroma cells in either healthy or atretic follicles. Moreover, these antibodies showed neither specific binding to the luteal body, oviduct, uterus, testis, liver, kidney, adrenal gland, pancreas, stomach, small intestine, large intestine, spleen, thymus, brain, heart, lung or skeletal muscle (data not shown).

Western blotting analyses

Conventional Western blotting analysis revealed that two specific bands with molecular weights of 42 and 55 kD were observed on the nitrocellulose filters treated with PFG-5 antibody in the homogenized samples of granulosa cell membrane prepared from healthy follicles, and that one band with molecular weights of 42 kD were observed on the filters treated with PFG-6 antibody. In the granulosa cell samples prepared from atretic follicles, a specific band with a molecular weight of 55 kD was observed on the nitrocellulose filters treated with PFG-5, but the band of 42 kD disappeared (data not shown). No positively stained bands were detected in the homogenized samples of the luteal body, oviduct, uterus, testis, liver, kidney, adrenal grand, pancreas, stomach, small intestine, large intestine, spleen, thymus, brain, heart, lung, adrenal gland, thyroid gland or skeletal muscle (data not shown).

Then, the antigens on the granulosa cells prepared from healthy antral follicles were characterized by 2D-Western blotting. Cell membrane fractions of the granulosa cells prepared from healthy and atretic fol-

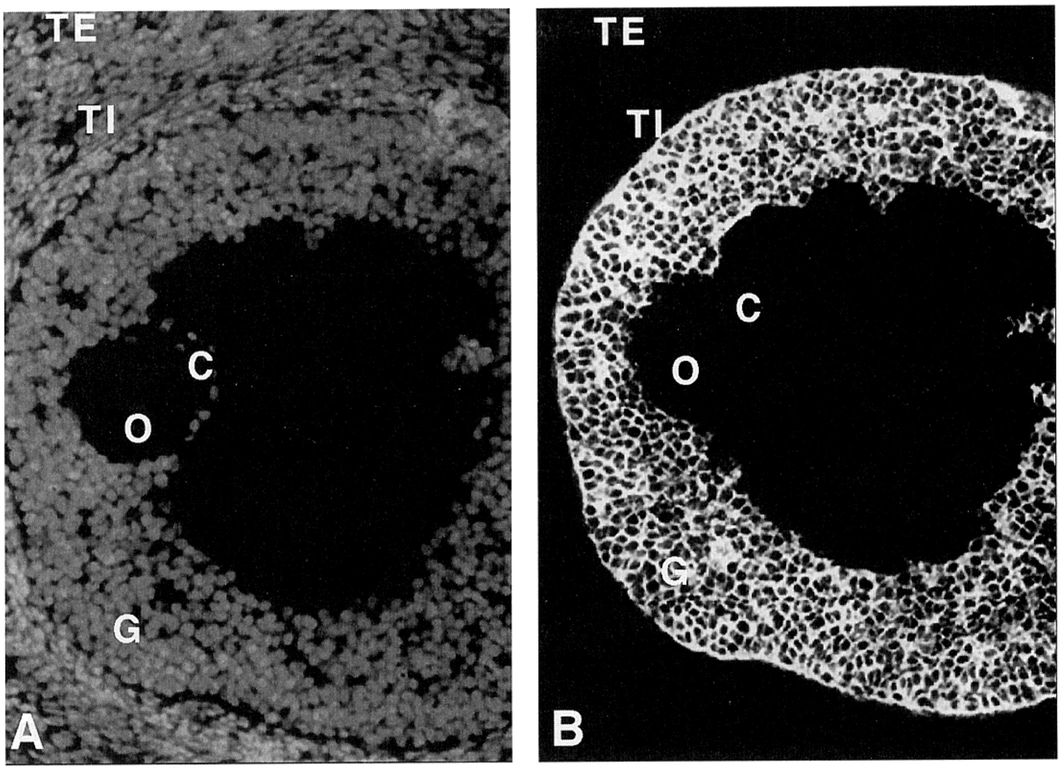

Fig. 2. Composite confocal images of healthy follicles. Frozen ovarian section of porcine ovary was stained with Hoechst 33258 to visualize the cell nuclei (A). The same section was stained with PFG-5 antibody and with FITC-conjugated anti-mouse IgM, to show the distribution of granulosa cell membrane antigens (B). The follicle was optically sectioned at 0.5 μm and five serial images were generated using the confocal microscope. PFG-5 antibody showed strong fluorescent staining on granulosa cells (G). No fluorescent staining of cumulus cells (C), oocyte (O), theca interna (TI) or externa (TE) layers was observed. × 200.

licles were subjected to 2D-PAGE. Representative 2D-PAGE results of healthy and atretic follicle samples are shown in Fig. 3. Protein spots in secondary slab-gel were detected by Coomassie brilliant blue staining (Fig. 3A and B). Representative results of 2D-Western blotting analysis on nitrocellulose membranes were shown in Fig. 3C-F. In healthy follicle samples, the two specific spots (42 kD, pI 5.2 and 55 kD, pI 5.9; named PFG-6 and PFG-5 antigens, respectively) of PFG-5 were demonstrated (Fig. 3C), and one specific spot (42 kD, pI 5.2; named PFG-6 antigen) of PFG-6 was detected (Fig. 3E). In the samples of atretic follicles, a spot of PFG-5 antigen was seen (Fig. 3D), but the spot of PFG-6 antigen was disappeared (Fig. 3D and F).

Granulosa cell apoptosis mediated by the monoclonal antibody

In the hybridoma screening procedure, granulosa cell-killing activity of the monoclonal antibodies was assessed by a apoptotic cell determination kit as described above. After selection of hybridoma clones, *in vitro* granulosa cell apoptosis mediated by the selected antibody was confirmed by assessment of TUNEL staining, nuclear morphology and DNA electrophoretic analysis. The isolated granulosa cells prepared from healthy follicles were co-cultured with concentrations varying from 0.0001 to 1,000 μg/ml of PFG-5 and/or PFG-6, antibodies for 3 to 72 h at 37°C. After incubation, apoptotic cells were determined. Dose-dependent changes in apoptosis inducels by the monoclonal antibodies were shown in Fig. 4, and representative photographs of TUNEL staining and agarose gel electrophoresis of cellular DNA were shown in Figs. 5 and 6, respectively. No apoptotic cells were detected in the isolated granulosa cells cultured with vehicle or with PFG-6 antibody (Fig. 4). When the isolated granulosa cells were cultured with at least 0.01 μg/ml PFG-5 antibody for more than 6 h, apoptotic cells were detected. As shown in Fig. 5, no TUNEL-positive

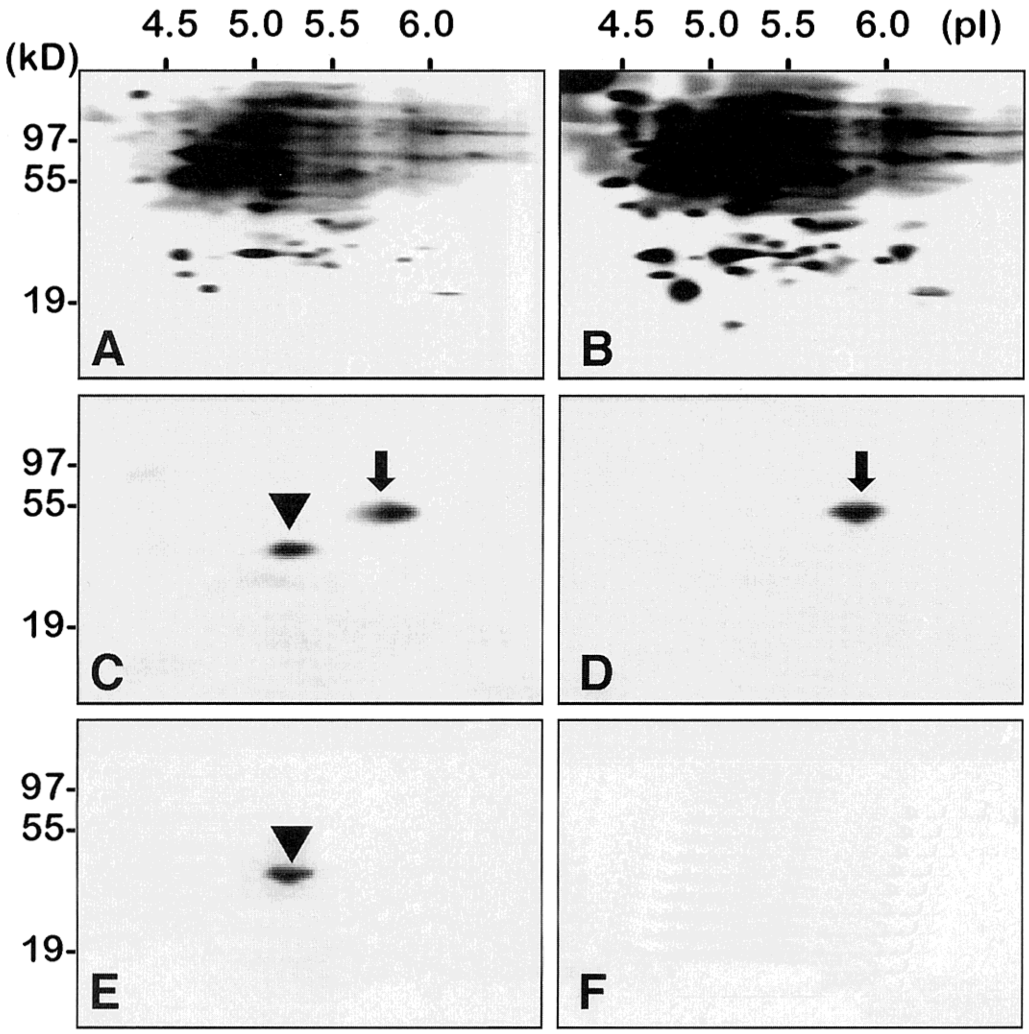

Fig. 3. Representative results of two-dimensional (2D) Western blotting analysis of granulosa cell-membrane antigens recognized by PFG-5 (C and D) and PFG-6 (E and F) antibodies. Granulosa cell membrane fractions prepared from healthy (A, C and E) and atretic (B, D and F) granulosa cells were separated by 2D-PAGE. Separated protein spots in gels were detected by Coomassie brilliant blue (A and B). After electrophoresis, the protein spots were transferred onto nitrocellulose sheets and the granulosa cell antigens were visualized by PFG-5 and PFG-6 antibodies. In healthy follicle samples, two specific spots (42 kD, pI 5.2 and 55 kD, pI 5.9; named PFG-6 and PFG-5 antigens: arrowhead and arrow, respectively) of PFG-5 antibody (C) were observed, and a spot of 42 kD, pI 5.2 (arrowhead) of PFG-6 antibody (E) was seen. In the samples of atretic follicles, the specific spot of 42 kD, pI 5.2 disappeared (D and F), and a spot of 55 kD, pI 5.9 antigen (arrow) was seen (D).

cells were observed in the isolated granulosa cells cultured with vehicle (10 μl/ml; Fig. 5A; vehicle control) or 100 μg/ml PFG-6 antibody (Fig. 5C) for 12 h, while many TUNEL-positive round nuclei and small condensed nuclear fractions (apoptotic bodies; a morphological hallmark of apoptotic cell death) were observed in the isolated granulosa cells cultured with 0.01 μg/ml PFG-5 antibody for 12 h (Fig. 5B). Interestingly, when the cells were co-cultured with 1 μg/ml of PFG-5 antibody and 100 μg/ml of PFG-6 antibody (PFG-5/PFG-6), no TUNEL-positive cells were observed (Fig. 5D). Thus, apoptotic cell death induced by PFG-5 antibody was inhibited by PFG-6 antibody. After incubation with PFG-5 and/or PFG-6 antibodies, DNA samples of these isolated granulosa cells were electrophoresed in 2% agarose gels. DNA samples of the isolated cells cultured with vehicle

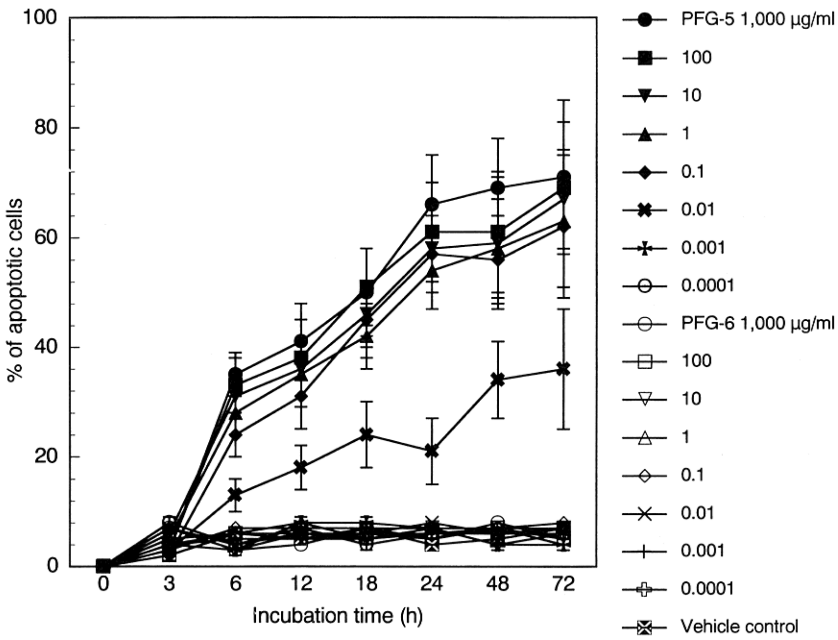

Fig. 4. Dose-dependency in apoptosis inducels by PFG-5 and PFG-6 antibodies. No apoptotic cells were detected in the isolated granulosa cells cultured with vehicle or with PFG-6 antibody. When the cells were cultured with at least 0.01 μg/ml PFG-5 antibody for more than 6 h, apoptotic cells were detected.

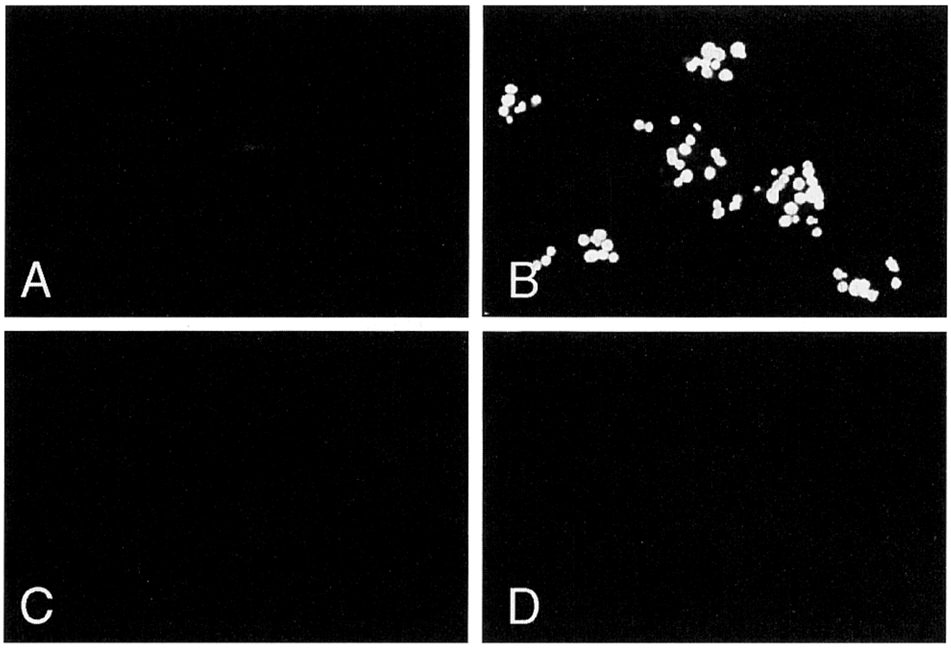

Fig. 5. Fluorescence photomicrographs of the cultured granulosa cells stained by the TUNEL method to assess apoptotosis. The granulosa cells prepared from healthy follicles were cultured with 10 μl/ml of vehicle (A; control), or with 0.01 μg/ml PFG-5 antibody (B) or 100 μg/ml PFG-6 antibody (C) for 12 h at 37°C. No TUNEL-positive cells were observed in the control (A). In PFG-5 antibody-treated cells, many TUNEL-positive round nuclei and small condensed nuclear fractions (apoptotic bodies) were observed (B), but no positive cells were seen among those cultured with PFG-6 antibody (C). When the cells were co-cultured with both 1 and 100 μg/ml of PFG-5 and PFG-6 antibodies, respectively, no positive cells were seen (D).

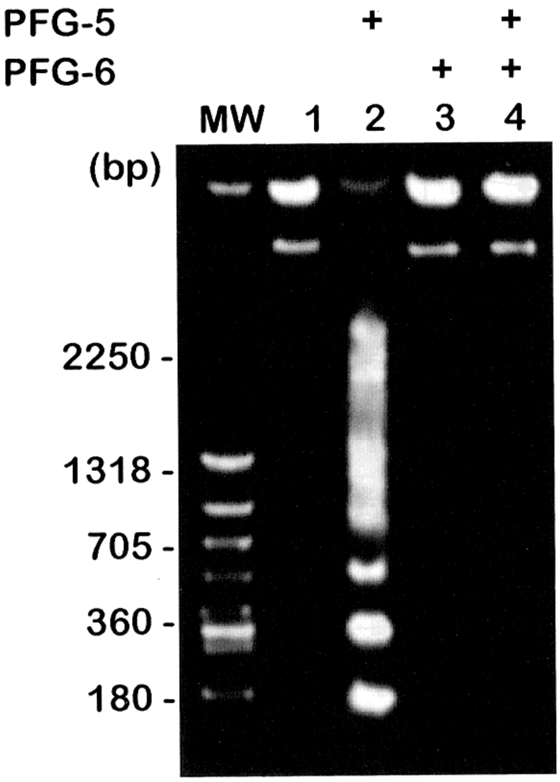

Fig. 6. Electrophoretic analysis of DNA fragments in DNA samples prepared from cultured granulosa cells. The isolated granulosa cells were cultured with 0.01 μg/ml PFG-5 or 100 μg/ml PFG-6 (lanes 2 and 3, respectively) for 12 h at 37°C, and then equal amounts of DNA samples prepared from cultured granulosa cells were electrophoresed. DNA samples from vehicle control cells displayed no DNA ladder formation on electrophoresis (lane 1). The DNA from PFG-5-treated granulosa cells displayed a ladder pattern (lane 2), but no such ladder pattern was seen in the DNA samples prepared from cells treated with PFG-6 (lane 3). When the cells were co-cultured with 1 and 100 μg/ml of PFG-5 and PFG-6 (lane 4), respectively, no ladder pattern was seen. Molecular weight markers (lane MW) are indicated on the left side of the figure.

displayed no ladder pattern on electrophoresis (Fig. 6 lane 1). PFG-6 antibody-incubated DNA sample displayed a ladder pattern (biochemical hallmark of apoptosis; Fig. 6 lanes 2), but no such ladder pattern was seen in the DNA samples prepared from PFG-6 antibody (Fig. 6 lane 3), PFG-5/PFG-6 co-incubated cells (Fig. 6 lanes 4).

Discussion

In the present study, we selected two hybridoma clones, named PFG-5 and PFG-6, producing monoclonal IgM and IgG antibodies, respectively, against cell-membrane proteins of granulosa cells. Immunohistochemical staining and conventional Western blotting analysis revealed that PFG-5 and PFG-6 antibodies showed specific binding to granulosa cells of ovarian follicles, but no specific binding to cumulus cells, oocytes, theca internal cells or theca externa cells. Moreover, these antibodies had no binding to the luteal body, oviduct, uterus, testis, liver, kidney, adrenal gland, pancreas, stomach, small intestine, large intestine, spleen, thymus, brain, heart, lung, adrenal gland, thyroid gland or skeletal muscle. 2D-Western blotting analysis revealed that the antibodies specifically recognized two cell membrane proteins named PFG-5 and PFG-6 antigens. PFG-5 antigen with a molecular weight of 55 kD and isoelectric point of 5.9 was detected in the granulosa cells of both healthy and atretic follicles, but PFG-6 antigen with a molecular weight of 42 kD and isoelectric point of 5.2 disappeared in atretic follicles. Abundant PFG-6 antigen expression was noted in the granulosa cells of healthy follicles. Immunohistochemical staining confirmed that PFG-5 and PFG-6 antigens were present only in the cell membrane fraction of follicular granulosa cells. Moreover, PFG-5 antibody but not PFG-6 antibody induced apoptotic cell death in cultured granulosa cells prepared from healthy antral follicles, and co-incubation with PFG-6 antibody inhibited PFG-5 antibody inducible apoptosis.

Previously, we generated a unique IgM monoclonal antibody, named PFG-1 antibody, and 2D-Western blotting analysis showed that this antibody recognized a cell membrane protein with a molecular weight of 55 kD and isoelectric point of 5.9 (named PFG-1 antigen) of porcine granulosa cells [27, 28]. Similarly to PFG-5 antigen, PFG-1 antigen was immunochemically and immunohistochemically detected only in the granulosa cells. PFG-1 antibody induced apoptotic cell death in cultured granulosa cells prepared from healthy follicles, and so PFG-1 antigen is considered to be a new cell death receptor. These findings indicated that PFG-1 antigen is the same membrane protein as PFG-5 antigen.

Fas/APO-1/CD95, a member of the TNF-receptor (TNF-R) superfamily, is a transmembrane protein that mediates apoptosis in a variety of lymphoid and tumor cells through Fas-ligand and Fas binding [36–38]. Fas mRNA is expressed in the thymus, liver, heart, lung and ovary [38, 39], but the physiological and pathological roles of the Fas-ligand and Fas system in the ovaries are not well understood. In rodent ovaries, Fas is expressed in follicular granulosa cells and mediates granulosa cell apoptosis in ovarian follicle atresia [40, 41]. Immunohistochemical staining of rat ovaries revealed intense positive immunostaining for Fas-ligand and Fas in granulosa cells of small and medium antral follicles with atresia, and intense Fas-ligand staining was evident in the theca interna cells of healthy small antral follicles [40, 42]. In *lps* mice with hereditary abnormality of Fas, extramly accumulation of follicles and luteal bodies were shown [41]. These observations indicated that Fas-ligand may be the signal which induces granulosa cell apoptosis during atresia in rodent ovaries. In mouse luteal bodies, which contain luteal cells, stromal cells, endothelial cells, fibroblasts and surface epithelial cells, Fas is expressed abundantly in surface epithelial cells and mediates apoptosis of the surface epithelial cells, and Fas involves with leuteolysis [41, 43].

Recently, *in vitro* studies demonstrated that other receptors (angiotensin II type 2 receptor, gonadotropin-releasing hormone receptor etc.) mediate follicular atresia [44, 45], but the *in vivo* physiological roles of these receptors in selection of atretic folliculus have not been determined. TNF also can induce apoptosis in a variety of tumor cells, and the TNF-R, a transmembrane protein, can also mediate apoptosis [46]. The molecular weights of Fas and TNF-R are 45–46 [38–40, 47] and 65 kD [46], respectively. As described above, the molecular weights of the granulosa cell-surface antigens recognized by PFG-5 and PFG-6 were 42 and 55 kD. Fas was immunohistochemically detected in the granulosa cells and luteal cells of both healthy and atretic follicles in rodent ovaries, but TNF-R was not detected in ovarian follicles or luteal bodies [15, 27, 28, 41]. However, PFG-5 antigen visualized histochemically by both PFG-5 was only detected in the granulosa cells. Based on their biochemical and histochemical characteristics, PFG-5 antigen is different from the known apoptosis-mediating receptors, Fas or TNF-R, and is considered to be a novel cell death receptor expressed specifically on the granulosa cells. The physiological properties of PFG-5 antigen, however, are not well understood. In the present study, we showed that PFG-5 antibody but not PFG-6 antibody can induce the apoptotic cell death of porcine granulosa cells in primary culture. It is interesting that the cultured granulosa cells were not killed by PFG-5 antibody in the presence PFG-6 antibody. As described above, PFG-5 antigen is considered to be a cell death receptor. Abundant expression of PFG-6 antigen was noted in granulosa cells of healthy follicles, and no expression was demonstrated in granulosa cells of atretic follicles. Thus, PFG-6 antigen plays as a survival receptor. Recently, TNF-related apoptosis-inducing ligand (TRAIL, also called Apo2 ligand), which is a novel CD95 ligand (Fas-ligand) homologous cytotoxic cytokine, was shown to belong to the TNF family, and to activate rapid apoptosis in various tumor cells [48–51]. TRAIL induces apoptosis upon binding to its cytoplasmic death domain-containing receptors, named death receptor 4 and 5 (DR4 and DR5, respectively). Both DR4 and DR5 independently bind to their specific ligand, TRAIL, and engage the caspase cascade to induce apoptosis similarly to Fas and TNF-R [52]. Moreover, two additional TRAIL binding membrane proteins, named decoy receptor 1 and 2 (DcR1 and DcR2, respectively), were identified [53, 54]. These decoy receptors are glycophospholipid-anchored cell surface proteins, lack functional cytoplasmic death domains, and act as antagonist decoy receptors that inhibit TRAIL-signaling. Apoptosis inducing receptors and decoy receptors are expressed on the same tumor cells [53, 54]. It is considered that overexpression of decoy receptor on the surface of tumor cells inhibits apoptotic cell death induced by TRAIL. Thus, a cell-surface mechanism exists for the regulation of cellular responsiveness to pro-apoptotic stimuli. The present results summarized that PFG-6 antigen is abundantly expressed on the surface of granulosa cells of healthy follicles and inhibits an apoptotic signal induced by ligand-like PFG-5 antibody, and that PFG-6 antigen plays as a modulator of PFG-5 antigen which transmits an apoptotic signal. As these properties of PFG-6 antigen are similar to the decoy receptors in TRAIL/DcR1 and DcR2 system, we presumed that PFG-6 antigen acts as a decoy receptor.

We hypothesize that PFG-5 antigen is a novel cell death receptor and a member of the TNF-R superfamily, and that PFG-6 antigen plays as a decoy receptor involved in regulation of granulosa cell survival and death. As these receptors may play an important role in control of ovarian functions, detailed biochemical studies should be performed. In our laboratory, PFG-5 and PFG-6 antibodies have been used to screen for cell death receptors on the granulosa cell membrane and to identify the cell death ligands binding to these receptors. Thus, these antibodies will be useful and sensitive probes to investigate the cell death receptors on the granulosa cell membrane and their natural ligands, to elucidate cell surface mechanisms for the regulation of apoptosis, and to define the intercellular pathway of apoptotic signal transduction in granulosa cells of porcine ovaries.

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