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Caspase-3 Activation is Associated with Granulosa Cell Apoptosis during Follicular Atresia in Porcine Ovary

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돼지 폐쇄난포내 과립세포의 자연세포사 시 캐스파제-3의 활성화

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ABSTRACT : Ovarian follicular atresia in mammals is finely regulated by gonadotropins and sex steroid hormones. It is well known that granulosa cell pyknosis is a common cytological feature of atretic follicles in the ovary. The present study hypothesized that granulosa cell pyknosis during follicular atresia might be related to apoptotic process and associated with caspase-3 activation. Healthy (normal) and atretic follicles were isolated from porcine ovaries based on macro-morphological criteria. Isolated follicles were either processed for histological observation or used for collection of granulosa cells by aspiration. Hoechst 33258 staining of the cells showed a significantly higher number of fragmented nuclei, a typical morphological feature of apoptotic cell, in granulosa cells from atretic follicles than those from healthy follicles. In addition, the rate of cell death was significantly higher in granulosa cells from atretic follicles than healthy follicles, as measured by flow-cytometric cell cycle analysis. *In situ* detection of apoptotic cells by TUNEL revealed that apoptosis was mostly restricted to granulosa cells in follicles. Theca cells were TUNEL-negative. Finally, it has been shown by caspase-3 activity assay that granulosa cells from atretic follicles retain a higher caspase-3 activity compared to healthy follicles. Taken together, it is suggested that granulosa cell degeneration during folliclar atresia occurs by caspase-3-dependent apoptotic fashion.

Key words : Caspase-3, Apoptosis, Granulosa cell, Atresia, Ovary, Pig.

요 약 : 포유류 난포의 폐쇄 과정은 매우 정교한 내분비적 조절작용에 의해 일어나며, 이 과정중에 발생하는 난포 내 과립세포의 퇴화는 핵응축 현상을 동반하는 것으로 알려져 있다. 본 연구는 핵응축 현상과 관련하여 돼지 난소 내 폐쇄난포의 과립세포가 퇴화 시 동반되는 세포 사멸이 아포토시스의 과정에 의해서 일어나는지의 여부와 아포토시스 관련 주요 단백질 분해 효소인 캐스파제-3과 연관된 세포 사멸 기전과 관련이 있는지에 대해서 조사하고자 하였다. 돼지 난소로부터 정상 및 폐쇄난포를 분리하고 이들을 대상으로 조직학적으로 아포토시스 발생 여부를 확인하였고 난포로부터 각각 얻어진 과립세포를 대상으로 하여 아포토시스 과정으로 사멸하는 세포의 형태 및 캐스파제-3의 활성을 관찰 및 측정하였다. 폐쇄 중 돼지 난포 내 과립세포에서 핵의 분절이 흔히 관찰되었고, 유세포 측정기를 이용하여 세포 사멸율을 측정해 본 결과 사멸하는 세포의 수가 폐쇄난포의 과립세포에서 정상난포보다 매우 유의하게 높은 것으로 나타났다. 난포 조직 내 아포토시스 세포는 과립세포에 국한되어 관찰되었으며 협막세포에서는 아포토시스가 관찰되지 않았다. 최종적으로 캐스파제-3의 활성을 정상 및 폐쇄 난포에서 분리한 세포에서 측정해 본 결과 폐쇄난포의 과립세포에서 정상난포보다 유의하게 높은 활성을 보였다. 이와 같은 결과를 종합해 볼 때, 돼지 난포의 폐쇄 중 과립세포의 퇴화는 아포토시스 과정에 의해 일어나며, 캐스파제-3의 활성에 의존적인 것으로 사료된다.

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INTRODUCTION

In mammals, the growth and development of an ovarian follicle proceed uninterruptedly until the follicle either ovu-

lates or undergoes a degenerative process known as atresia (Richards, 1980; Ryan, 1981). It is estimated that greater than 99% of follicles become atretic in the human ovary and about 77% in the mouse(Byskov, 1978). Despite the overwhelming occurrence of atresia in the ovary, the cellular and physiological events underlying this phenomenon remain poorly understood(Ryan, 1981). However, several studies have demonstrated that apoptosis is involved with the degeneration of granulosa cells in atretic follicles(Hughes & Gorospe, 1991; Jolly *et al.*, 1994; Murdoch, 1995).

To date, two apoptotic pathways have been proposed: One is involved with cell death receptors(TNF-R or Fas) in cellular membrane, in which binding of ligand to the receptor activates initiator caspase-8 and in turn proteolytically activates downstream effector caspases(caspase-3 and caspase-7) (Boatright & Salvesen, 2003). The other is associated with mitochondrial alterations such as cytochrome c release from mitochondrial membrane, which followed by activation of effector caspase via caspase-9 activation(Plesnila, 2004). In these both pathways, caspase-3 appears to be commonly activated as an effector protease.

Granulosa cell apoptosis in porcine atretic follicles was known to be accompanied by the appearance of DNA fragmentation, the hallmark of apoptosis(Tilly *et al.*, 1991). However, it is unknown whether the granulosa cell apoptosis occurs through the activation of caspase-3. Therefore, in the present study, we have demonstrated that the granulosa cell death in atretic follicles exhibits typical morphological and biochemical evidences of apoptosis including caspase-3 activation.

MATERIALS AND METHODS

1. Follicle Isolation from Porcine Ovaries

Ovaries were collected at local abattoirs around Busan and Seoul area and immediately transported to laboratory within 1 hr for subsequent follicle isolation. Follicles only sized from 4~6 mm were dissected, and classified as healthy and atretic follicles based on morphological characteristics(Moor *et al.*, 1978; Maxson *et al.*, 1985). Healthy (nonatretic) follicles were characterized by translucent, well-vascularized follicular wall, whereas atretic follicles exhibited an opaque surface traversed by few or no blood vessels. Ovaries with cysts or hemorrhagic follicles were not used in this study. After isolation, follicular fluid was aspirated from each follicle for the collection of granulosa cells by centrifugation(250g, 5min).

2. Fluorescent Staining of Apoptotic Nuclei

Isolated granulosa cells were smeared on slide glass and dried in air for 1 min. Cells were treated with mild hypertonic solution(distilled water: phosphate buffered saline = 1:1) for 10 min. Then, cells were prefixed in 50% solution of fixative(methanol:actic acid = 1:1) for 5 min and followed by fixation with neat fixative for 10 min. After fixation, cells were stained with Hoechst 33258(0.1mg/mL) for 10 min and observed with fluorescence microscope(Olympus BX-50, Tokyo).

3. Cell Death Assay by Flowcytometry

Cells were fixed with 95% ethanol containing 1% Triton X-100 and stained with propidium iodide(PI) containing 1 μg/mL RNase A for 30 min. The histograms were recorded on a FACS caliber instrument with an analysis software(Beckman Coulter, MI, USA). Cells belongs to sub-G1 population was considered as apoptotic cells and the percentage of each phase of cell cycle was determined.

4. Tissue Preparation and *In Situ* Localization of Apoptotic Cells

Follicles were fixed in 4% neutral buffered formaldehyde(pH 7.4) and embedded in paraffin. Sections(6mm) were mounted on slides, deparaffinized, hydrated, and treated for 30min at 37℃ with proteinase-K(10 μg/mL in 20mM Tris and 2mM $CaCl_2$, pH 7.4). DNA 3'-end labeling with nonradioactive digoxigenin-dideoxy-UTP(dig-ddUTP) was performed after incubation for 10 min in terminal trasferase buffer(200mM potassium cacodylatate, 25 mM Tris, 0.25mg/mL BSA, and 5mM $CoCl_2$, pH 6.6) at room temperature. Terminal transferase(1U/mL), dig-ddUTP (5mM) and ddATP were added in fresh buffer and incubated at 37℃ in a humidified chamber for 1 hr. Washed in Tris buffer, the sections were incubated with blocking buffer(100 mM Tris, 150mM NaCl(pH 7.5), and 0.5% blo-

cking reagent; Roche) for 30min at RT before the addition of antidigoxigenin antibodiy conjugated to alkaline phosphatase. After incubation with the antibody(1:1,000 in 0.5 % blocking reagent, 100mM Tris, and 150mM NaCl, pH 7.5) at RT for 2 hr in a humidified chamber, the slides were equilibrated in alkaline phosphatase buffer(100mM Tris, 100mM NaCl, and 50mM $MgCl_2$, pH 9.5) before the addition of substrates(337.5mg/mL nitroblue tetrazolium and 175 μg/mL 5-bromo-4-chloro-3-inodyl-phosphate) for alkaline phosphatase. In negative control slides, terminal transferase enzyme reaction was omitted, and the remaining procedures were all the same as above described.

5. Caspase-3 Activity Assay

Cells were dissolved in lysis buffer [30mM HEPES, 1 mM DDT, 1mM EDTA, 0.1% CHAPS, (pH 7.4)]. 30 μg of proteins were incubated with a fluorogenic caspase-3 substrate(Ac-DEVD-AMC from Calbiochem, La Jolla, CA, USA) in assay buffer [100mM NaCl, 50 mM HEPES, 10 mM DDT, 1mM EDTA, 0.1% CHAPS, 10% glycerol, (pH 7.4)] at 37 ℃. If required, 0.5 μM caspase-3 specific inhibitor(Ac-DEVD-CHO) was added to the reaction. The fluorescence was monitored by fluorometer(Packard Bioscience, MA, USA) with excitation at 360nm and emission at 530nm every 30min.

6. Statistical Analysis

Data were expressed as means±SD and analyzed by Student *t*-test. A p value of less than 0.05 was considered to be significant.

RESULTS

1. Nuclear Fragmentation and Apoptosis in Porcine Aranolosa Cells

To evaluate whether porcine granulosa cells are exhibiting typical morphologic characteristics of apoptosis, granulosa cells were stained with Hoechst 33258 dye and their nuclear morphology was observed by fluorescence microscopy. As shown in Fig. 1, while granulosa cells obtained from healthy follicles retained intact nuclear morphology (Fig. 1A), apoptotic fragmented nuclei were seen in the cells from atretic follicles(Fig. 1B).

Next, the apoptotic proportion in granulosa cells was determined by flow cytometry. Cell cycle histograms of granulosa cells recovered from the porcine healthy and atretic follicles were shown in Fig. 2. The percentage of granulosa cells from atretic follicles in A_0(sub-G_1) phase (Fig. 2B) was significantly($p<0.0001$) higher than that of healthy(Fig. 2A). And the percentages of A_0 phase in both porcine follicles were inversely proportional to that of G0/G1 phase(Fig. 2C).

2. *In Situ* Detection of Apoptotic Cells on Porcine Healthy and Atretic Follicles

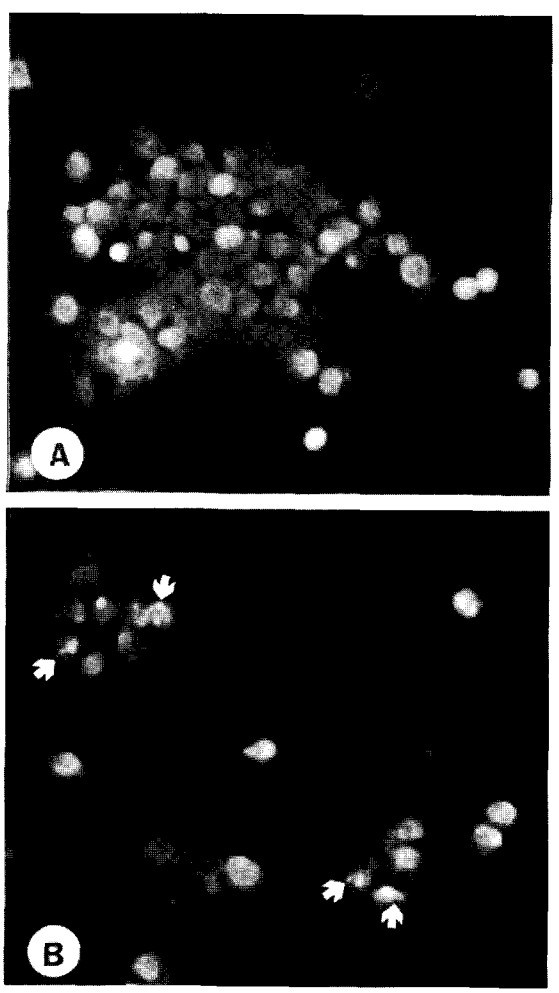

Fig. 1. Nuclear fragmentation in granulosa cells from porcine ovary. Cells were stained with Hoechst 33258 fluorescence dye. A, granulosa cells from healthy follicles; B, granulosa cells from atretic follicles. Arrows indicate fragmented nuclei. Original magnification, ×500.

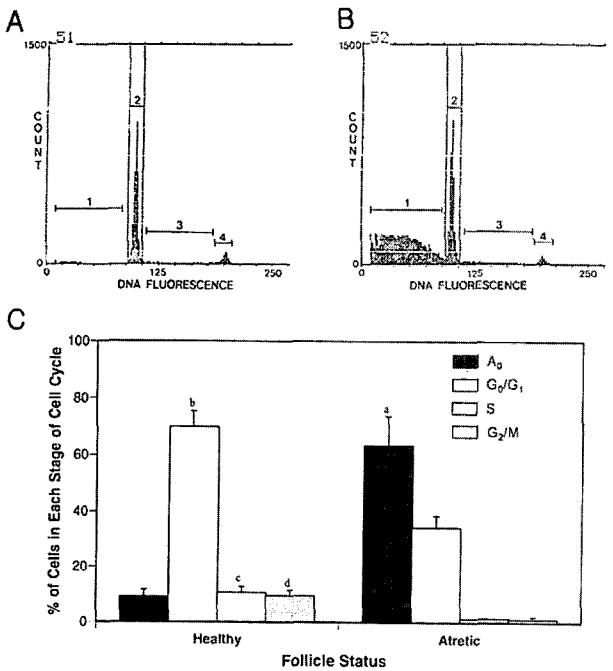

Fig. 2. Flowcytometirc analysis of cell death and cycle with granulosa cells from porcine ovary. Isolated cells were stained with propidium iodide and applied to flowcytometer. Sub-G_1 (A_0) phase was considered as dead cells. A, a representative flowcytogram of granulosa cells from healthy follicles; B, a representative flowcytogram of granulosa cells from atretic follicles; C, a graph showing percentage of cells in each phase of cell cycle. In flowcytogram, 1, 2, 3 and 4 indicate sub-G_1 (A_0), G_0/G_1, S and G_2/M phase, respectively.

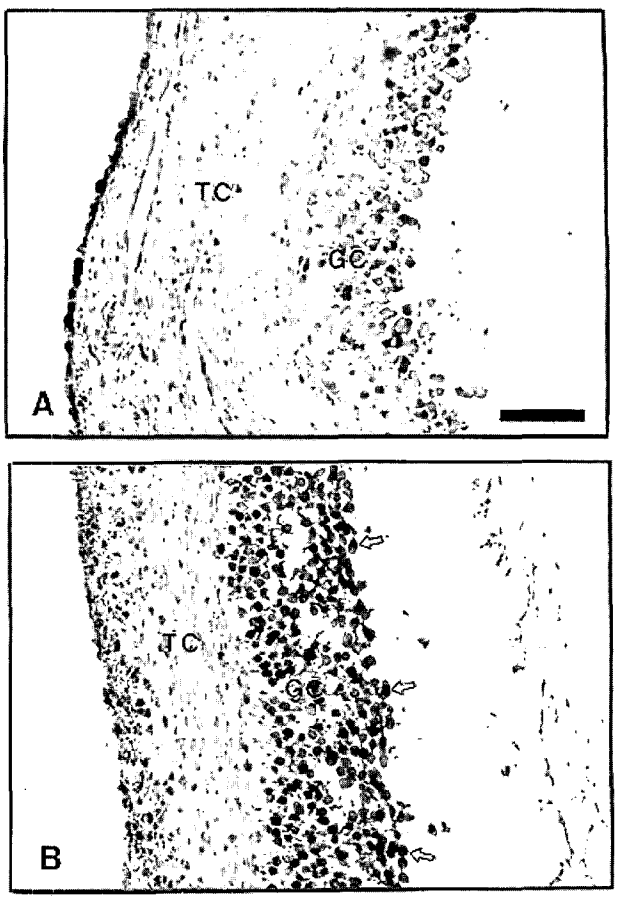

Fig. 3. *In situ* detection of apoptotic cells in follicles from porcine ovary. Paraffin embedded follicles were stained with TUNEL. A, Healthy follicle; B, Atretic follicle; TC and GC indicate to theca cells and granulosa cells, respectively. Arrows point to TUNEL-positive cells. Bar, 100 μm, Original magnification, ×500.

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling(TUNEL) is a very useful tool for localizing the apoptotic cells on tissues. In the present study, we have localized the apoptotic cells in isolated ovarian follicles. In healthy follicles, TUNEL-positive cells were not detected in granulosa cells as well as theca cells(Fig. 3A). In contrast, TUNEL-positive cells were frequently seen in granulosa cells of atretic follicles but not in theca cells(Fig. 3B).

3. Caspase-3 Activation in Porcine Granolosa Cells

In order to investigate whether granulosa cell apoptosis in porcine atretic follicles is caspase-3-dependent, we have measured caspase-3 activity in granulosa cell lysates from healthy and atretic follicles using a fluorogenic caspase-3 substrate(Ac-DEVD-AMC). Caspase-3 activity was significantly higher in granulosa cells from atretic follicles than those from healthy follicles. This activity was suppressed in the presence of the specific caspase-3 inhibitor, Ac-DEVD-CHO(Fig. 4).

DISCUSSION

In the present study, we have demonstrated that granulosa cell apoptosis during follicular atresia in porcine ovary is associated with caspase-3 activation. In porcine follicles, atresia is often characterized by a reduction in estrogen concentration in follicular fluids(Maxson *et al.*, 1985). It has been shown that granulosa cell apoptosis can be either suppressed by estrogen(Billig *et al.*, 1993), FSH (Chun *et al.*, 1994), EGF and bFGF(Tilly *et al.*, 1992) or

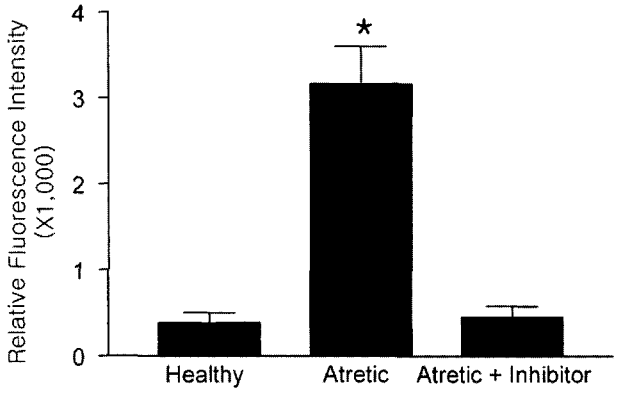

Fig. 4. Caspase-3 acitivity in granulosa cells of porcine ovarian follicles. For caspase-3 activity assay, Ac-DEVD-AMC and Ac-DEVD-CHO were used as a fluorogenic substrate and a specific inhibitor, respectively. Cell lysates(30 μg of proteins) were added to the reaction and the fluorescence was monitored by fluorometer with excitation at 360 nm and emission at 530 nm. *, $p<0.05$ compared with healthy follicles.

inhibited by androgens(Billig *et al.*, 1993) and GnRH(Billig *et al.*, 1994). Although the exact molecular mechanism (s) by which these hormones modulate granulosa cell apoptosis during follicular atresia is still unclear, several important apoptotic proteins have been implicated in granulosa cells of atretic follicles. These include Fas/FasL system(Kim *et al.*, 1998), IAPs(Li *et al.*, 1998), DNase I (Boone *et al.*, 1995), caspase-3(Boone and Tsang, 1998) and p53(Kim *et al.*, 1999). In these studies, gonadotropins and estrogen clearly suppressed the apoptosis in granulosa cells, indicating that these hormones are acting as survival factors for granulosa cells *in vivo*.

Although the role of apoptotic proteins in granulosa cells during follicular atresia is relatively well known in rat ovary(Boone *et al.*, 1995; Kim *et al.*, 1998; Li *et al.*, 1998), in porcine granulosa cells, only incidence of cell death has been demonstrated by flowcytometric analysis(Guthrie *et al.*, 1994, 1995). Therefore, the morphological and biochemical evidences(nuclear fragmentation and TUNEL) shown in this study are supporting the evidence of granulosa cell apoptosis in porcine atretic follicles. Consistent with the occurrence of apoptosis in rat ovary, *in situ* TUNEL on porcine ovarian follicles clearly showed that granulosa cells are mostly apoptotic in atretic follicles. These results confirm that, also in porcine ovary, granulosa cells not theca cells are the type of cells undergoing apoptosis in antral atretic follicles.

So far, fourteen caspases have been demonstrated in the apoptotic pathway cascade(Cryns & Yuan, 1998). Among these, caspase-3 is considered to be a major effector protease(Woo *et al.*, 1998; Porter & Janicke, 1999). The cleavage of caspase-3 from its pro form to its active form has been shown to be critical for its role in apoptosis(FernandesAlnemri & Alnemri, 1994). Caspase-3 cleavage can be triggered by active caspase-8, processed from its proform after Fas ligation(Stennicke *et al.*, 1998), or by caspase-9, processed from its pro form after increased ratios of heterodimerized Bax/Bcl-2 ratio(Li *et al.*, 1997). It has been shown in a number of cell types that, once activated, caspase-3 cleaves numerous cellular proteins associated with the cytoskeleton(Mashima *et al.*, 1997; Caulin *et al.*, 1997), cell cycle regulation(Chen *et al.*, 1997; Tan *et al.*, 1997), DNA repair(Tewari *et al.*, 1995), and DNA degradation (Sakahira *et al.*, 1998). Among these substrates, it has been suggested that active caspase-3 causes caspase-activated deoxyribonuclease(CAD) to be dissociated from its inhibitor protein, inhibitor of CAD(Sakahira *et al.*, 1998; Wolf *et al.*, 1999). The liberated CAD then is thought to translocate into the nucleus to cause DNA fragmentation(Enari *et al.*, 1998). We have shown here that caspase-3 activity is significantly increased in granulosa cells from atretic follicles compared to healthy follicles, strongly suggesting granulosa cells die via a caspase-3-dependent mechanism.

Taken together, these results suggest that granulosa cell apoptosis during follicular atresia in porcine ovary represents typical characteristics of apoptosis, and further that caspase-3-dependent mechanism is required for granulosa cell apoptosis. In other system, caspase-8 and -9 are activated by ligation of Fas ligand to Fas and by an increase in the Bax/Bcl-2 ratio, respectively. As yet, there is uncertainty about whether granulosa cell apoptosis in porcine atretic follicles begins with ligation of Fas ligand to Fas, changes in the Bcl-2 family, or both. To answer this important issue, we currently are studying the possible activation of caspase-8, caspase-9, or both.

REFERENCES

Billig H, Furuta I, Hsueh AJW (1993) Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. Endocrinology 133:2204-2212.

Billig H, Furuta I, Hsueh AJW (1994) Gonadotropin-releasing hormone directly induces apoptotic cell death in the rat ovary: biochemical and *in situ* detection of deoxyribonucleic acid fragmentation in granulosa cells. Endocrinology 134:245-252.

Boatright KM, Salvesen GS (2003) Mechanisms of caspase activation. Curr Opin Cell Biol 15:725-731.

Boone DL, Tsang BK (1998) Caspase-3 in the rat ovary: localization and possible role in follicular atresia and luteal regression. Biol Reprod 58:1533-1539.

Boone DL, Yan W, Tsang BK (1995) Identification of a deoxyribonuclease I-like endonuclease in rat granulosa and luteal cell nuclei. Biol Reprod 53:1057-1065.

Byskov AG (1978) Follicular atresia. In: Jones RE (ed.), The vertebrate ovary: comparative biology and evolution. Plenum, New York, pp 533-562.

Caulin C, Salvesen GS, Oshima RG (1997) Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. J Cell Biol 138:1379-1394.

Chen L, Marechal V, Moreau J, Levine AJ, Chen J (1997) Proteolytic cleavage of the mdm2 oncoprotein during apoptosis. J Biol Chem 272:22966-22973.

Chun SY, Billig H, Tilly JL, Furuta I, Tsafriri A, Hsueh AJW (1994) Gonadotropin suppression of apoptosis in cultured preovulatory follicles: mediatory role of endogeneous insulin-like growth factor I. Endocrinology 135: 1845-1853.

Cryns V, Yuan J (1998) Proteases to die for. Genes Dev 12:1551-1570.

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S (1998) A caspaseactivated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391:43-50.

FernandesAlnemri T, Litwack G, Alnemri ES (1994) CPP-32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 betaconverting enzyme. J Biol Chem 269:30761-30764.

Guthrie HD, Cooper BS, Welch GR, Zakaria AD, Johnson LA (1995) Atresia in follicles grown after ovulation in the pig: measurement of increased apoptosis in granulosa cells and reduced follicular fluid Estradiol-17β. Biol Reprod 52:920-927.

Guthrie HD, Welch GR, Cooper BS, Zakaria AD, Johnson LA (1994) Flow cytometric determination of degraded deoxynuclei acid in granulosa cells to identify atretic follicles during preovulatory maturation in the pig. Biol Reprod 50:1303-1311.

Hughes FM Jr, Gorospe WC (1991) Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. Endocrinology 129:2415-2422.

Jolly PD, Tisdall DJ, Health DA, Lun S, McNatty KP (1994) Apoptosis in granulosa cells in regulation to steroid synthesis, cycle adenosine 3',5'-monophosphate response to follicle-stimulating hormone and luteinizing hormone, and follicular atresia. Biol Reprod 51:934-944.

Kim JM, Boone DL, Auyeung A, Tsang BK (1998) Granulosa cell apoptosis induced at the penultimate stage of follicular development is associated with increased levels of Fas and Fas ligand in the rat ovary. Biol Reprod 58:1170-1176.

Kim JM, Yoon YD, Tsang BK (1999) Involvement of the Fas/Fas ligand system in p53-mediated granulosa cell apoptosis during follicular development and atresia. Endocrinology 140:2307-2317.

Li J, Kim JM, Liston P, Li M, Miyazaki T, Mackenzie AE, Korneluk RG, Tsang BK (1998) Expression of inhibitor of apoptosis proteins(IAPs) in rat granulosa cells during ovarian follicular development and atresia. Endocrinology 139:1321-1328.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf1/caspase9 complex initiates an apoptotic protease cascade. Cell 91:479-489.

Mashima T, Naito M, Noguchi K, Miller DK, Nicholson DW, Tsuruo T (1997) Actin cleavage by CPP32/apopain during the development of apoptosis. Oncogene 14:

1007-1012.

Maxson WS, Haney AF, Schomberg DW (1985) Steroidogenesis in porcine atretic follicles: loss of aromatase activity in isolated granulosa and theca. Biol Reprod 33:495-501.

Moor RM, Hay MF, Dott HM, Cran DG (1978) Macroscopic identification and steroidogenic function of atretic follicles in sheep. J Endocrinol 77:309-318.

Murdoch WJ (1995) Programmed cell death in preovulatory ovine follicle. Biol Reprod 53:8-12.

Plesnila N (2004) Role of mitochondrial proteins for neuronal cell death after focal cerebral ischemia. Acta Neurochir Suppl 89:15-19.

Porter AG, Janicke RU (1999) Emerging roles of caspase3 in apoptosis. Cell Death Differ 6:99-104.

Richards JS (1980) Maturation of ovarian follilces: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. Physiol Rev 60:51-89.

Ryan RJ (1981) Follicular atresia: some speculations of biochemical markers and mechanisms. In: Schwarz NB, HunzickerDunn M (ed.), Dynamics of ovarian function. Raven Press, New York.

Sakahira H, Enari M, Nagata S (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391:96-99.

Stennicke HR, Jurgensmeier JM, Shin H, Deveraux Q, Wolf BB, Yang X, Zhou Q, Ellerby HM, Ellerby LM, Bredesen D, Green DR, Reed JC, Froelich CJ, Salvesen GS (1998) Procaspase3 is a major physiologic target of caspase8. J Biol Chem 273:27084-27090.

Tan X, Martin SJ, Green DR, Wang JY (1997) Degradation of retinoblastoma protein in tumor necrosis factor and CD95 induced cell death. J Biol Chem 272:9613-9616.

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(AD-Pribose) polymerase. Cell 81:801-809.

Tilly JL, Billig H, Kowalski KI, Hsueh AJ (1992) Epidermal growth factor and basic fibroblast growth factor suppress the spontaneous onset of apoptosis in cultured rat ovarian granulosa cells and follicles by a tyrosine kinease dependent mechanism. Mol Endocrinol 6:1942-1950.

Tilly JL, Kowalski KI, Johnson AL, Hsueh AJW (1991) Involvement of apoptosis in ovarian follicular atresia and post ovulatory regression. Endocrinology 129:2799-2801.

Wolf BB, Schuler M, Echeverri F, Green DR (1999) Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor45/inhibitor of caspaseactivated DNase inactivation. J Biol Chem 274:30651-30656.

Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW, Mak TW (1998) Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev 12:806-819.