Journal of Reproduction and Development, Vol. 52, No. 6, 2006

—Mini Review—

The Regulation of Ovarian Granulosa Cell Death by Pro- and Anti-apoptotic Molecules

Fuko MATSUDA-MINEHATA[1)], Naoko INOUE[2)], Yasufumi GOTO[1)] and Noboru MANABE[1)]

[1)]Research Unit for Animal Life Sciences, Animal Resource Science Center, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Ibaraki-Kasama 319-0206 and [2)]Laboratory of Animal Morphology and Function, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

Abstract. In the mammalian ovary, follicular development and atresia are closely regulated by cell death and survival-promoting factors, including hormones (gonadotropins) and intraovarian regulators (gonadal steroids, cytokines, and intracellular proteins). Several hundred thousand primordial follicles are present in the mammalian ovary; however, only a limited number of primordial follicles develop to the preovulatory stage and ovulate. The others, more than 99% of follicles, will be eliminated via a degenerative process known as "atresia". The endocrinological regulatory mechanisms involved in follicular development and atresia have been characterized to a large extent, but the precise temporal and molecular mechanisms involved in the regulation of these events have remained largely unknown. Recent studies suggest that the apoptosis of ovarian granulosa cells plays a major role in follicular atresia. In this review, we provide an overview of development and atresia of follicles, and apoptosis of granulosa cells in mammals.
Key words: Anti-apoptotic factor, Apoptosis, Follicular atresia, Granulosa cell, Porcine ovary, Pro-apoptotic factor

(**J. Reprod. Dev. 52:** 695–705, 2006)

Apoptosis plays a significant role in almost all physiological functions in vertebrate and invertebrate species. It is a form of cell death essential for elimination of cells that are damaged, senescent, potentially harmful, or no longer useful [1]. The major features of apoptosis are internucleosomal DNA fragmentation, cell shrinkage, plasma membrane blebbing, and the formation of apoptotic bodies. Stimulation by death ligands or deprivation of key survival-promoting growth factors is the main contributor to apoptosis, while stress inducers, including drugs, toxicants, oxidative stress, and radiation, are also known to cause apoptosis.

Studies have revealed that apoptosis also plays a crucial role in maintaining the reproductive apparatus. During follicular growth and development, more than 99% of follicles disappear, primarily due to apoptosis of granulosa cells [2–5] (Fig. 1). Both the biochemical and morphological characteristics of apoptosis have been observed in the granulosa cells of atretic follicles [6–8]. Apoptotic stimuli and intracellular signal transduction pathways involved in the apoptosis of granulosa cells remain to be determined, and investigators are studying potential triggers of apoptosis and how intracellular apoptotic signals are transmitted in granulosa cells. To date, many

Accepted for publication: July 10, 2006
Published online: August 23, 2006
Correspondence: N. Manabe (e-mail: amanabe@mail.ecc.u-tokyo.ac.jp)

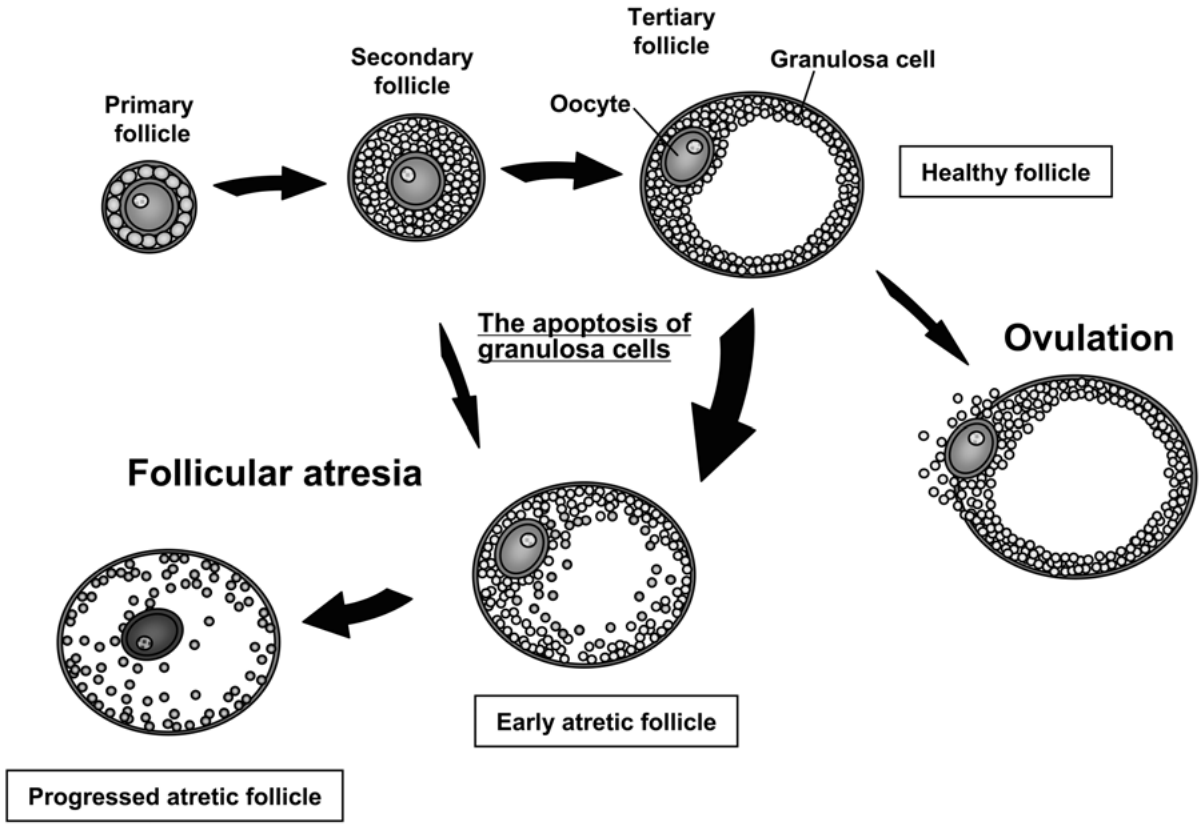

Fig. 1. Development and atresia of follicles in mammalian ovaries. During adult life, a number of primordial follicles begin growing during each estrus cycle. Primary follicles (with a monolayer of follicular epithelial/granulosa cells) develop into secondary follicles (with stratified granulosa cells but without an antrum) and subsequently into tertiary follicles (with a follicular antrum). During follicular growth and development, most follicles undergo a degenerative process called atresia and less than 0.1% ovulate. Based on recent findings, it has been suggested that follicular atresia is caused by the apoptosis of granulosa cells. Follicles in which granulosa cells begin to separate from the follicular wall are called early atretic follicles, and follicles in which granulosa cells are completely disconnected are called progressed atretic follicles.

apoptosis-related factors have been implicated in follicular atresia, including death ligands and receptors, intracellular pro- and anti-apoptotic molecules, cytokines, and growth factors.

Apoptosis in Mammalian Cells

Death receptors and ligands

Death receptors, which bind to cell membranes and are located on the cell surface, constitute a subfamily within the tumor necrosis factor receptor superfamily (TNFRsf) that has a cytoplasmic death domain (DD) necessary for the activation of apoptosis. These receptors are trimerized and then bind to death ligands, which act as a trigger for apoptosis. Cell death ligand-receptor systems known in mammals include the tumor necrosis factor-α (TNF-α) and TNF-α receptors (TNFRs), Fas ligand (FasL) and Fas (CD95, APO-1, TNFRsf6), and TNF-α-related apoptosis-inducing ligand (TRAIL) and TRAIL receptors [9, 10]. In most cases, the cell death receptor-mediated apoptotic signaling pathway is as follows. (1) Cell death ligands, which bind with cell membranes and are located at the cell surface, bind to the extracellular domain of trimerized cell death receptors, each of which contains an intracellular DD. (2) The DD of the death receptor binds with the DD of the adaptor protein (Fas-associated death domain: FADD) through a homophilic interaction. (3) An initiator caspase (procaspase-8; also called FLICE) binds to FADD through a homophilic interaction of the death effector domain (DED; the resulting complex

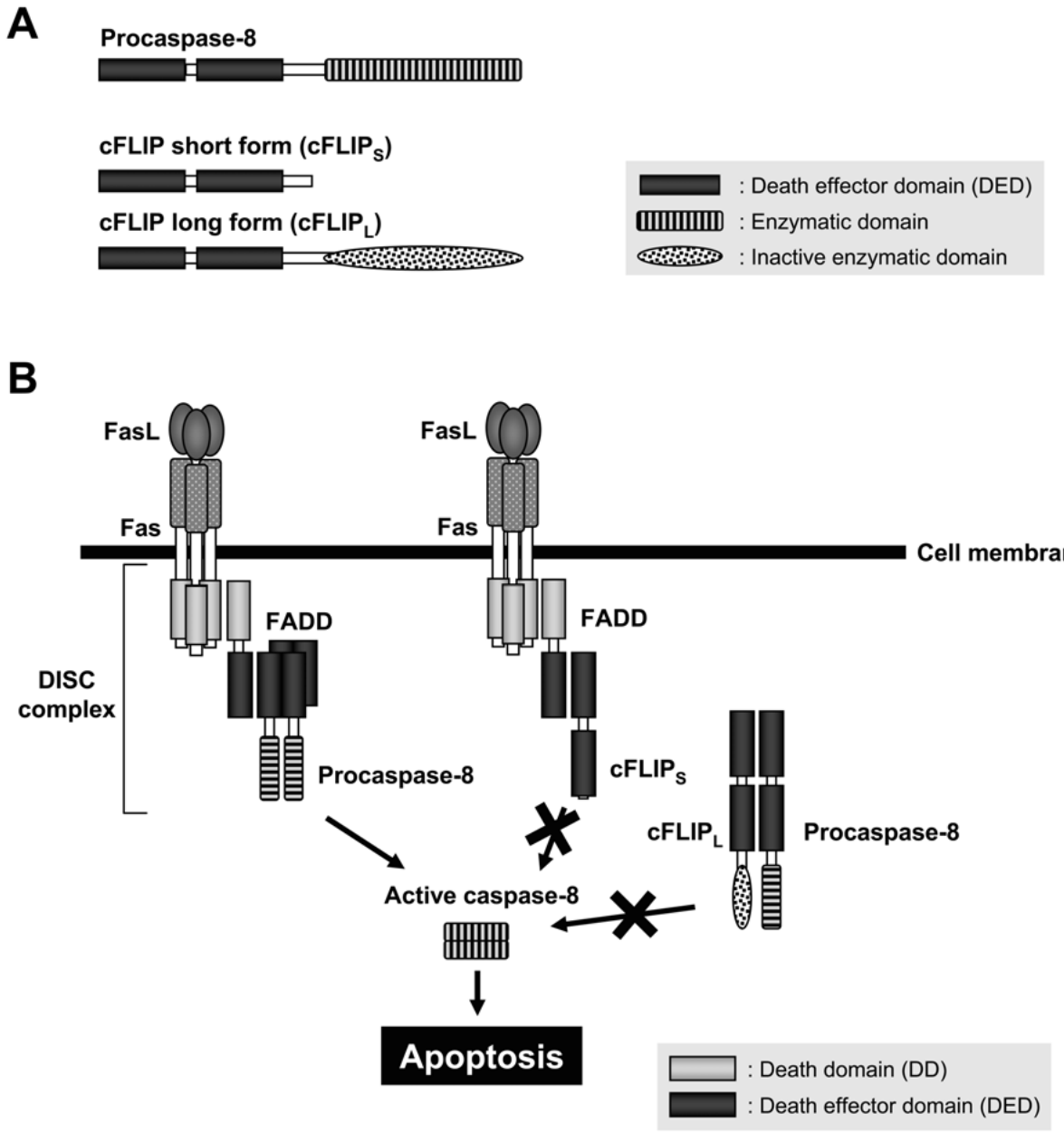

Fig. 2. (A) Schematic representation of procaspase-8, cFLIP short form (cFLIP$_S$), and cFLIP long form (cFLIP$_L$). Procaspase-8 consists of two death effector domains (DEDs) and an enzymatic domain. There are two DEDs in cFLIP$_S$, and there is an inactive enzymatic domain in cFLIP$_L$. (B) Regulation of Fas-mediated apoptosis by cFLIP. When the Fas ligand (FasL) binds to the extracellular region of Fas, the adaptor protein, Fas-associated death domain (FADD), binds to the intracellular region of Fas via the homophilic interaction of the death domains (DDs). Then, procaspase-8 binds to FADD through the homophilic interaction of the DEDs. Dimerized procaspase-8 is cleaved and active caspase-8 induces activation of downstream caspases, which results in apoptosis. Fas-signaling is blocked by cFLIP by binding to FADD or procaspase-8, which inhibits activation of caspase-8.

is called the death-inducing signaling complex: DISC). (4) Dimerization of procaspase-8 induces auto-proteolytic cleavage and activation. (5) The activated caspase-8 subsequently activates downstream caspases either directly ("type I") or *via* mitochondrial perturbation ("type II"), which results in apoptosis [11–15] (Fig. 2B). However, death ligand-receptor interaction does not

necessarily result in cell death, indicating the importance of intracellular inhibitors of the apoptotic signaling pathway.

Cellular FLICE-like inhibitory protein (cFLIP: also called CASH, Casper, CLARP, FLAME, I-FLICE, MRIT, or usurpin), a homologue of procaspase-8, is one of the intracellular proteins that interferes with the apoptotic effects of death ligands [16–22]. FLIP was first identified in several viruses as viral FLIP (vFLIP), which contains two DEDs that interact with FADD to avoid the host's apoptotic response [23]. There are two splicing variants of cFLIP, a short form ($cFLIP_S$) and a long form ($cFLIP_L$). The structure of $cFLIP_S$ is very similar to vFLIP, containing two DEDs, while $cFLIP_L$ contains an additional pseudo-enzymatic domain that is similar to the enzymatic domain of procaspase-8 but lacks enzymatic activity [20] (Fig. 2A). Recent reports have indicated both $cFLIP_S$ and $cFLIP_L$ to be important regulators of apoptosis that block death ligand-inducible apoptosis, mainly FasL-Fas signaling, by competing with procaspase-8 and inhibiting the activation of caspase-8 [24, 25] (Fig. 2B).

The Bcl-2 family

As described above, the signal after DISC's formation differs between cell types. Two classifications, type I and type II, have been established for death ligand-mediated apoptotic signaling [15]. In type I cells, a large amount of caspase-8 is activated at the DISC, closely followed by rapid cleavage of caspase-3. In contrast, the activation of caspase-3 in type II cells occurs mainly downstream of the mitochondrion. The main factors involved in mitochondrial dysfunction are the B cell/lymphoma-2 (Bcl-2) family proteins, including inhibitors (Bcl-2, Bcl-X_L, etc.) and promoters (Bid, Bax, Bak, Bad, Bim, etc.) of apoptosis. The activated mitochondria release cytochrome c, and the binding of cytochrome c with apoptosis-activating factor 1 (Apaf-1) causes the processing of procaspase-9 into the mature enzyme and activates downstream caspases, like caspase-3 [26].

Growth factors and signal transduction by protein kinases

In addition to death ligand-receptor signaling, the absence of growth factors or cytokines induces apoptosis. The cellular proliferation that is induced by growth factors or cytokines can only occur in the presence of distinct survival-promoting signals. Cells that receive signals to proliferate in the absence of survival-promoting signals do not proliferate, but instead die by apoptosis. A key cell survival pathway linked to growth factor receptors is the phosphatidylinositol 3-kinase (PI3-K)/Akt phosphorylation cascade [27]. A number of studies have confirmed that PI3-K is activated when recruited to the cytoplasmic surface of the plasma membrane following the activation of growth factor receptors by ligands or via direct interaction with the Ras protooncogene [28]. Activation of PI3-K results in activation of a broad spectrum of downstream kinases, including Akt (also known as PKB). Under homeostatic conditions, growth factor-activated Akt serves to phosphorylate, and thereby regulate, proteins that function to maintain the basic needs of the cell, such as transport and oxidation of glucose to produce energy [29, 30].

Recently, the FOXO (forkhead box, class O) subfamily of forkhead transcription factors (FOXO1/FKHR, FOXO3a/FKHRL1, and FOXO4/AFX) has been identified as a direct target of PI3-K. FOXO transcription factors are directly phosphorylated by Akt, resulting in binding to 14-3-3 proteins, nuclear export, and inhibition of transcription. However, in the absence of Akt-mediated phosphorylation, FOXO transcription factors can translocate to the nucleus and increase the gene expression of pro-apoptotic regulatory factors such as FasL and Bim [31–33]. It has also been reported that phosphorylated Akt upregulates the expression of cFLIP, which results in inhibition of apoptosis [34, 35].

Follicular Development and Atresia in the Mammalian Ovary

Follicular development during fetal and adult life

During embryogenesis, primordial germ cells migrate from the yolk sac to the genital ridge and proliferate. Then, the somatic cells, called follicular epithelial cells (granulosa cells), enclose the germ cells to form primordial follicles. After mitosis, the first meiotic division begins in germ cells (primary oocytes). The primary oocyte is arrested at the diplotene stage of meiosis until the surrounding follicles leave the primordial stage (primordial follicles) and then starts to grow to reach ovulation.

Approximately 5 million primordial follicles are present in both ovaries 10 days after birth in sows (1.2, 4, and 1 million primordial follicles in cows, women, and mice, respectively) [36–40]. During the period a sow is fertile, a maximum of 1,600 oocytes (less than 0.14% of all primary oocytes) will ovulate, and all others will disappear.

After puberty, a number of primordial follicles start to grow during each estrus cycle in adult females. Initiation of follicular growth involves endocrinological factors, mainly follicle stimulating hormone (FSH), and local modulating factors from granulosa cells, theca cells, stromal-interstitial cells, and oocytes. Primary follicles (follicles with a monolayer of follicular epithelial/granulosa cells) develop into secondary follicles (follicles with stratified granulosa cells but without an antrum) and subsequently into tertiary follicles (follicles with a follicular antrum). Due to a large increase in the proliferation of granulosa cells and an increase in the size of the antrum, tertiary follicles show an exponential rate of growth [41]. In the oocyte, meiosis then restarts and the first polar body divides. Finally, selected follicles burst, and the oocytes ovulate (Fig. 1).

Regulation of follicular development and atresia

With the increase in serum FSH concentrations at the start of the estrous cycle, follicles produce more estrogen and inhibin via granulosa cells. As a feedback mechanism, the inhibin causes a decrease in FSH secretion, and therefore the remaining small follicles undergo atresia. Experiments with FSH receptor (FSHR) knockout mice and hypophysectomized rodents have shown that FSH is essential for formation of the antrum in secondary follicles and postantral follicular development in tertiary follicles [42]. Active immunization against inhibin increased the number of ovulations in sows [43, 44], and injection of inhibin into the ovarian bursa of immature rats increased the number of medium-sized antral follicles [45], indicating that inhibin acts to prevent follicular development. Although atresia can occur at any time during follicular development, the majority of follicles become atretic during the early antral stage of development [40]. The transition from preantral to antral follicles occurs after exposure of the granulosa cells to gonadotropin. Then, differentiation of the granulosa cells is initiated, and this renders them susceptible to apoptosis. Granulosa cells from antral, but not preantral, follicles contain endogenous DNase I [46, 47]. However, the presence of this endonuclease is not sufficient to cause apoptosis; a signal to activate DNase I and induce cell death is required. Although the exact signals, receptors, and intracellular signaling pathways leading to apoptosis within granulosa cells are unclear, many molecules are likely involved, including follicle survival factors [such as FSH, insulin-like growth factor-I (IGF-I), interleukin-1β (IL-1β), epidermal growth factor (EGF), Bcl-2, Bcl-X_L, etc.] and atretogenic factors (inhibin, Bax, FasL, TNF-α, caspase, etc.) [48]. Follicular growth and development and atresia seem to depend upon a sophisticated balance between these survival and atretogenic molecules.

Regulation of Follicular Atresia by Apoptosis of Granulosa Cells

Death receptors and follicular atresia

The FasL-Fas system is the most characterized apoptotic signaling machinery in granulosa cells. In many species including humans, mice, rats, cows, and sows, both FasL and Fas are expressed in granulosa cells, and apoptosis is inducible by FasL-Fas signaling *in vitro* [49–58]. In human females, the granulosa cells of antral follicles express Fas during the early stages of atresia, and the levels of Fas expression increase as atresia progresses [51]. In addition, treatment of female mice with Fas-activating antibody promotes apoptosis of granulosa cells and follicular atresia [50, 53], indicating a pro-apoptotic function of FasL-Fas signaling *in vivo*.

TNF-α is known to induce both cell death and cell proliferation. TNF-α exerts its effects by binding either to TNF receptor (TNFR)-1, which contains a DD, and stimulating apoptotic signaling or to TNFR2, which lacks a DD and acts as a survival/anti-apoptotic and/or proliferating factor [59, 60]. In primary cultured granulosa cells, TNF-α can induce both proliferation and death [61, 62]. However, based on expression experiments in porcine ovaries, TNF-α seems to act as a cell survival factor since levels of TNFR2 and TNF receptor associated factor-2 (TRAF2: activator of apoptosis initiated by TNF-α signaling) decrease during follicular atresia [63].

Although few studies have been conducted on

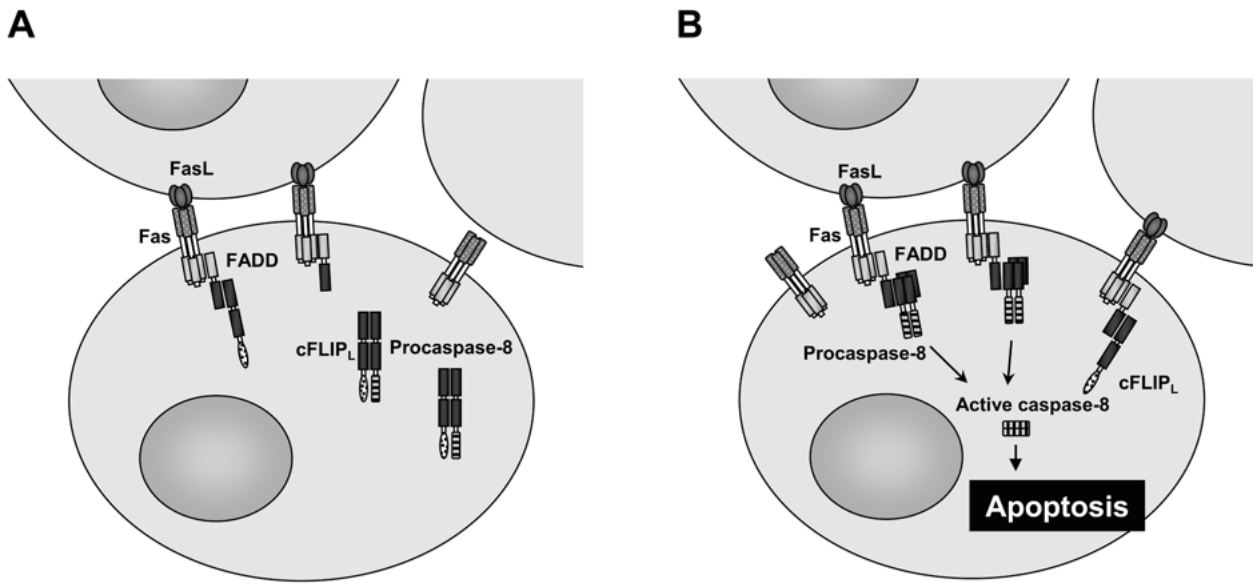

Fig. 3. Working hypothesis on granulosa cell survival in healthy follicles (A) and atretic follicles (B). (A) Although the cell death ligand and receptor (ex. FasL and Fas) are expressed and interact on granulosa cells, subsequent apoptotic signaling is blocked by cFLIP$_L$. As a result, apoptosis of the granulosa cells is avoided and the follicle is kept healthy. (B) When the cFLIP$_L$ expression level is low, FasL-Fas interaction causes cleavage of procaspase-8 and subsequent apoptotic signaling. As a result, apoptosis of the granulosa cells is induced and the follicle undergoes atresia.

the possible role of TRAIL in contrast to FasL and TNF-α, TRAIL and its receptor [TRAIL-decoy receptor 1 (DcR1)] have been indicated to induce apoptosis of granulosa cells based on their levels of expression and activity in porcine ovaries [64, 65].

Recently, we found that cFLIP$_S$ and cFLIP$_L$ are expressed in porcine granulosa cells and determined their mRNA sequences initially in pig species [66]. The homology of porcine cFLIP vs. human and murine cFLIP was very high (more than 75% for both the mRNA and amino acid levels), and we have proposed that cFLIP also has cell survival-promoting effects in pig species. As described above, cFLIP is known to inhibit Fas-mediated apoptosis, and the FasL-Fas system is well-characterized as a pro-apoptotic signal in granulosa cells. In porcine granulosa cells, the expression of FasL and Fas increases during atresia, although both proteins are also expressed in healthy pre-antral and antral follicles [67]. It has been suggested that the factor(s) that blocks FasL-Fas-mediated apoptotic signaling is essential for maintaining granulosa cells and keeping follicles healthy.

To determine the role of cFLIP in ovarian granulosa cells, we first examined the expression of cFLIP using porcine ovaries by RT-PCR and Western blotting. The mRNA and protein of cFLIP$_L$ were highly expressed in the granulosa cells of healthy follicles and decreased during atresia. The mRNA levels of cFLIP$_S$ in granulosa cells were low and showed no changes among the stages of follicular development. Furthermore, the protein level of cFLIP$_S$ was extremely low. By *in situ* hybridization, cFLIP$_L$ was found to be abundant in the granulosa cells of healthy follicles in comparison with those of atretic follicles. Immunohistochemical analyses demonstrated that the cFLIP protein is highly expressed in the granulosa cells of healthy follicles but weakly expressed in those of atretic follicles [68]. We presumed that cFLIP, especially cFLIP$_L$, plays an anti-apoptotic role in the granulosa cells of healthy follicles in pig ovaries.

Since the anti-apoptotic activity of porcine cFLIP (pcFLIP) had not been determined, we next examined the effect of pcFLIP on survival using granulosa-derived cell lines. A human ovarian granulosa tumor cell line, KGN [69], and porcine granulosa-derived cell line, JC-410 [70], were used. KGN cells transfected with pcFLIP$_S$ or pcFLIP$_L$ vectors survived induction of Fas-mediated

apoptosis, while almost all cells transfected with empty vector died, indicating the anti-apoptotic activity of pcFLIP in granulosa cells. When both $cFLIP_S$ and $cFLIP_L$, or $cFLIP_L$ only, were suppressed by small interfering RNA (siRNA), the viability of the JC-410 cells decleased significantly [71]. We conclude that porcine cFLIP functions as an anti-apoptotic factor in granulosa-derived cells.

These findings strongly suggest that cFLIP acts as a survival-promoting factor in granulosa cells and determines whether porcine ovarian follicles survive or undergo atresia (Fig. 3). Considering the results indicating an apoptotic effect of $cFLIP_S$ in rat primary cultured granulosa cells [62], cFLIP may be the key regulating factor of ovarian granulosa cell death in mammals.

Bcl-2 family members in granulosa cell death

Bcl-2 family proteins also appear to regulate the apoptosis of granulosa cells. The role of Bcl-2 in ovarian apoptosis is supported by several experimental findings. The numbers of follicles decreased in Bcl-2-deficient mice [72], and excessive expression of Bcl-2 leads to decreased follicular apoptosis and atresia [73, 74]. Bax-deficient mice have abnormal follicles with an excessive number of granulosa cells [75], and Bax expression is strong in atretic follicles as compared with the healthy follicles of human ovaries [76]. In murine and porcine granulosa cells, caspase-9 and Apaf-1 have been indicated to cause follicular atresia [77, 78]. These findings strongly suggest that mitochondria play a critical role in the execution of apoptosis in granulosa cells, which are categorized as type II apoptotic cells.

Growth factors and signal transduction in granulosa cell apoptosis

IGF-I plays a critical role in the development of ovarian follicles in many species. In the rat follicle culture model, treatment with IGF-I prevents spontaneous onset of apoptosis [79]. IGF-I knockout mice are infertile, as follicular development is arrested at the small antral stage and mature tertiary, Graafian follicles are not produced [80, 81]. In primary cultured porcine granulosa cells, IGF-I promotes proliferation and suppresses apoptosis [82–84]. It has been demonstrated that treatment of granulosa cells with IGF-I stimulates the activities of PI3-K and Akt, with IGF-I driving phosphorylation [85–87], indicating that IGF-I plays an anti-apoptotic role in granulosa cells by maintaining PI3-K-Akt signaling. The expression of FOXO1, a pro-apoptotic transcription factor, is regulated by Akt in the granulosa cells of rats and mice and decreases during follicular growth [88]. In addition, transcription of the FOXO1 gene and phosphorylation of the FOXO1 protein in granulosa cells is regulated by IGF-I and FSH [88–90]. FOXO3a is also necessary for ovarian follicular development, as FOXO3a null mice are infertile due to impairment of the early stages of follicular growth [91, 92]. The functions of forkhead transcription factors may be critical, and further experiments are necessary to determine the precise molecular mechanisms behind follicular growth and development.

In addition to IGF-I, EGF, basic fibroblast growth factor (bFGF), and IL-1β are also characterized as cytokines that inhibit the apoptosis of granulosa cells. EGF decreases the apoptosis of porcine granulosa cells [84]. In cultured rat ovarian granulosa cells and follicles, EGF and bFGF suppress apoptosis [93, 94]. IL-1β inhibits apoptosis in cultured rat preovulatory follicles [95].

Conclusion

Through the efforts of many researchers, a number of factors regulating the apoptosis of ovarian granulosa cells have been identified. However, the dominant factor(s) that determines follicular development or atresia *in vivo* remains unclear. Solving this problem is essential for elucidating how the reproductive system develops in vertebrates and may improve the low rate of gestation for *in vitro* fertilization in domestic animals and humans. If the mechanisms by which follicles are selected can be understood clearly, methods for selecting healthy oocytes or techniques to improve damaged oocytes may be established.

Acknowledgements

This work was supported by a Grant-in-Aid for Creative Scientific Research (13GS0008) to N. M., by a Grant-in-Aid for Scientific Research (B) (18380164), Exploratory (18658105) and (S) (16108003 and 18108004) to N. M., and by Research

Fellowships for Young Scientists to F. M-M. from the Japan Society for the Promotion of Science. We are grateful to Drs. Takashi Miyano (Kobe University, Kobe, Japan) and Kazuhiro Sakamaki for their critical advice.

References

1. **Schwartzman RA, Cidlowski JA**. Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocr Rev* 1993; 14: 133–151.
2. **Hughes FM Jr, Gorospe WC**. Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. *Endocrinology* 1991; 129: 2415–2422.
3. **Tilly JL, Kowalski KI, Johnson AL, Hsueh AJ**. Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. *Endocrinology* 1991; 129: 2799–2801.
4. **Kaipia A, Hsueh AJ**. Regulation of ovarian follicle atresia. *Annu Rev Physiol* 1997; 59: 349–363.
5. **Jiang JY, Cheung CK, Wang Y, Tsang BK**. Regulation of cell death and cell survival gene expression during ovarian follicular development and atresia. *Front Biosci* 2003; 8: 222–237.
6. **Palumbo A, Yeh J**. In situ localization of apoptosis in the rat ovary during follicular atresia. *Biol Reprod* 1994; 51: 888–895.
7. **Manabe N, Imai Y, Ohno H, Takahagi Y, Sugimoto M, Miyamoto H**. Apoptosis occurs in granulosa cells but not cumulus cells in the atretic antral follicles in pig ovaries. *Experientia* 1996; 52: 647–651.
8. **Boone DL, Carnegie JA, Rippstein PU, Tsang BK**. Induction of apoptosis in equine chorionic gonadotropin (eCG)-primed rat ovaries by anti-eCG antibody. *Biol Reprod* 1997; 57: 420–427.
9. **Ashkenazi A, Dixit VM**. Death receptors: signaling and modulation. *Science* 1998; 281: 1305–1308.
10. **Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP**. Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu Rev Immunol* 1999; 17: 331–367.
11. **Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM**. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 1995; 81: 505–512.
12. **Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM**. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 1996; 85: 817–827.
13. **Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, Peter ME**. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J* 1997; 16: 2794–2804.
14. **Nagata S**. Apoptosis by death factor. *Cell* 1997; 88: 355–365.
15. **Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME**. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 1998; 17: 1675–1687.
16. **Goltsev YV, Kovalenko AV, Arnold E, Varfolomeev EE, Brodianskii VM, Wallach D**. CASH, a novel caspase homologue with death effector domains. *J Biol Chem* 1997; 272: 19641–19644.
17. **Han DK, Chaudhary PM, Wright ME, Friedman C, Trask BJ, Riedel RT, Baskin DG, Schwartz SM, Hood L**. MRIT, a novel death-effector domain-containing protein, interacts with caspases and BclXL and initiates cell death. *Proc Natl Acad Sci USA* 1997; 94: 11333–11338.
18. **Hu S, Vincenz C, Ni J, Gentz R, Dixit VM**. I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD-95-induced apoptosis. *J Biol Chem* 1997; 272: 17255–17257.
19. **Inohara N, Koseki T, Hu Y, Chen S, Nunez G**. CLARP, a death effector domain-containing protein interacts with caspase-8 and regulates apoptosis. *Proc Natl Acad Sci USA* 1997; 94: 10717–10722.
20. **Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J**. Inhibition of death receptor signals by cellular FLIP. *Nature* 1997; 388: 190–195.
21. **Shu HB, Halpin DR, Goeddel DV**. Casper is a FADD- and caspase-related inducer of apoptosis. *Immunity* 1997; 6: 751–763.
22. **Srinivasula SM, Ahmad M, Ottilie S, Bullrich F, Banks S, Wang Y, Fernandes-Alnemri T, Croce CM, Litwack G, Tomaselli KJ, Armstrong RC, Alnemri ES**. FLAME-1, a novel FADD-like anti-apoptotic molecule that regulate Fas/TNFR1-induced apoptosis. *J Biol Chem* 1997; 272: 18542–18545.
23. **Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F, Mattmann C, Burns K, Bodmer JL, Schroter M, Scaffidi C, Krammer PH, Peter ME, Tschopp J**. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* 1997; 386: 517–521.
24. **Krueger A, Baumann S, Krammer PH, Kirchhoff S**.

FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Mol Cell Biol* 2001; 21: 8247–8254.
25. **Thome M, Tschopp J**. Regulation of lymphocyte proliferation and death by FLIP. *Nat Rev Immunol* 2001; 1: 50–58.
26. **Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A, Daugas E, Geuskens M, Kroemer G**. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J Exp Med* 1996; 184: 1331–1341.
27. **Datta SR, Brunet A, Greenberg ME**. Cellular survival: a play in three Akts. *Genes Dev* 1999; 13: 2905–2927.
28. **Cantley LC**. The phosphoinositide 3-kinase pathway. *Science* 2002; 296: 1655–1657.
29. **Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA**. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 1995; 378: 785–789.
30. **Kohn AD, Summers SA, Birnbaum MJ, Roth RA**. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J Biol Chem* 1996; 271: 31372–31378.
31. **Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME**. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 1999; 96: 857–868.
32. **Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L, Coffer PJ**. FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. *J Cell Biol* 2002; 156: 531–542.
33. **Stahl M, Dijkers PF, Kops GJ, Lens SM, Coffer PJ, Burgering BM, Medema RH**. The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. *J Immunol* 2002; 168: 5024–5031.
34. **Suhara T, Mano T, Oliveira BE, Walsh K**. Phosphatidylinositol 3-kinase/Akt signaling controls endothelial cell sensitivity to Fas-mediated apoptosis via regulation of FLICE-inhibitory protein (FLIP). *Circ Res* 2001; 89: 13–19.
35. **Panka DJ, Mano T, Suhara T, Walsh K, Mier JW**. Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. *J Biol Chem* 2001; 276: 6893–6896.
36. **Baker TG**. A quantitative and cytological study of germ cells in human ovaries. *Proc R Soc Lond B Biol Sci* 1963; 158: 417–433.
37. **Black JL, Erickson BH**. Oogenesis and ovarian development in the prenatal pig. *Anat Rec* 1968; 161: 45–55.
38. **Himelstein-Braw R, Byskov AG, Peters H, Faber M**. Follicular atresia in the infant human ovary. *J Reprod Fertil* 1976; 46: 55–59.
39. **Byskov AG**. Differentiation of mammalian embryonic gonad. *Physiol Rev* 1986; 66: 71–117.
40. **Hirshfield AN**. Development of follicles in the mammalian ovary. *Int Rev Cytol* 1991; 124: 43–101.
41. **Grant SA, Hunter MG, Foxcroft GR**. Morphological and biochemical characteristics during ovarian follicular development in the pig. *J Reprod Fertil* 1989; 86: 171–183.
42. **Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P**. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc Natl Acad Sci USA* 1998; 95: 13612–13617.
43. **Brown RW, Hungerford JW, Greenwood PE, Bloor RJ, Evans DF, Tsonis CG, Forage RG**. Immunization against recombinant bovine inhibin α subunit causes increased ovulation rates in gilts. *J Reprod Fertil* 1990; 90: 199–205.
44. **King BF, Britt JH, Esbenshade KL, Flowers WL, Sesti LA, Martin TL, Ireland JJ**. Ovulatory and endocrine responses after active immunization of gilts against a synthetic fragment of bovine inhibin. *J Anim Sci* 1993; 71: 975–982.
45. **Woodruff TK, Lyon RJ, Hansen SE, Rice GC, Mather JP**. Inhibin and activin locally regulate rat ovarian folliculogenesis. *Endocrinology* 1990; 127: 3196–3205.
46. **Boone DL, Yan W, Tsang BK**. Identification of a deoxyribonuclease I-like endonuclease in rat granulosa and luteal cell nuclei. *Biol Reprod* 1995; 53: 1057–1065.
47. **Boone DL, Tsang BK**. Identification and localization of deoxyribonuclease I in the rat ovary. *Biol Reprod* 1997; 57: 813–821.
48. **Hussein MR**. Apoptosis in the ovary: molecular mechanisms. *Hum Reprod Update* 2005; 11: 162–177.
49. **Quirk SM, Cowan RG, Joshi SG, Henrikson KP**. Fas antigen-mediated apoptosis in human granulosa/luteal cells. *Biol Reprod* 1995; 52: 279–287.
50. **Hakuno N, Koji T, Yano T, Kobayashi N, Tsutsumi O, Taketani Y, Nakane PK**. Fas/APO-1/CD95 system as a mediator of granulosa cell apoptosis in ovarian follicle atresia. *Endocrinology* 1996; 137: 1938–1948.
51. **Kondo H, Maruo T, Peng X, Mochizuki M**. Immunological evidence for the expression of the Fas antigen in the infant and adult human ovary during follicular regression and atresia. *J Clin Endocrinol Metab* 1996; 81: 2702–2710.
52. **Guo MW, Xu JP, Mori E, Sato E, Saito S, Mori T**. Expression of Fas ligand in murine ovary. *Am J Reprod Immunol* 1997; 37: 391–398.

53. **Sakamaki K, Yoshida H, Nishimura Y, Nishikawa S, Manabe N, Yonehara S**. Involvement of Fas antigen in ovarian follicular atresia and luteolysis. *Mol Reprod Dev* 1997; 47: 11–18.
54. **Kim JM, Boone DL, Auyeung A, Tsang BK**. Granulosa cell apoptosis induced at the penultimate stage of follicular development is associated with increased level of Fas and Fas ligand in the rat ovary. *Biol Reprod* 1998; 58: 1170–1176.
55. **Peng X, Maruo T, Matsuo H, Takekida S, Deguchi J**. Serum deprivation-induced apoptosis in cultured porcine granulosa cells is characterized by increased expression of p53 protein, Fas antigen and Fas ligand and by decreased expression of PCNA. *Endocr J* 1998; 45: 247–253.
56. **Kim JM, Yoon YD, Tsang BK**. Involvement of the Fas/Fas ligand system in p53-mediated granulosa cell apoptosis during follicular development and atresia. *Endocrinology* 1999; 140: 2307–2317.
57. **Vickers SL, Cowan RG, Harman RM, Porter DA, Quirk SM**. Expression and activity of the Fas antigen in bovine ovarian follicle cells. *Biol Reprod* 2000; 62: 54–61.
58. **Porter DA, Harman RM, Cowan RG, Quirk SM**. Relationship of Fas ligand expression and atresia during bovine follicle development. *Reproduction* 2001; 121: 561–566.
59. **Hsu H, Xiong J, Goeddel DV**. The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. *Cell* 1995; 81: 495–504.
60. **Boldin MP, Mett IL, Varfolomeev EE, Chumakov I, Shemer-Avni Y, Camonis JH, Wallach D**. Self-association of the "death domains" of the p55 tumor necrosis factor (TNF) receptor and Fas/APO1 prompts signaling for TNF and Fas/APO1 effects. *J Biol Chem* 1995; 270: 387–391.
61. **Prange-Kiel J, Kreutzkamm C, Wehrenberg U, Rune GM**. Role of tumor necrosis factor in preovulatory follicles of swine. *Biol Reprod* 2001; 65: 928–935.
62. **Xiao CW, Asselin E, Tsang BK**. Nuclear factor κB-mediated induction of Flice-like inhibitory protein prevents tumor necrosis factor α-induced apoptosis in rat granulosa cells. *Biol Reprod* 2002; 67: 436–441.
63. **Nakayama M, Manabe N, Inoue N, Matsui T, Miyamoto H**. Changes in the expression of tumor necrosis factor (TNF)α, TNFα receptor (TNFR) 2, TNFR-associated factor 2 in granulosa cells during atresia in pig ovaries. *Biol Reprod* 2003; 68: 530–535.
64. **Wada S, Manabe N, Nakayama M, Inoue N, Matsui T, Miyamoto H**. TRAIL-decoy receptor 1 plays inhibitory role in apoptosis of granulosa cells from pig ovarian follicles. *J Vet Med Sci* 2002; 64: 435–439.
65. **Inoue N, Manabe N, Matsui T, Maeda A, Nakagawa S, Wada S, Miyamoto H**. Roles of tumor necrosis factor-related ligand signaling pathway in granulosa cell apoptosis during atresia in pig ovaries. *J Reprod Dev* 2003; 49: 313–321.
66. **Goto Y, Matsuda-Minehata F, Inoue N, Matsui T, Maeda A, Manabe N**. Porcine (*Sus Scrofa*) cellular FLICE-like inhibitory protein (cFLIP): molecular cloning and comparison with the human and murine cFLIP. *J Reprod Dev* 2004; 50: 549–555.
67. **Inoue N, Maeda A, Matsuda-Minehata F, Fukuta K, Manabe N**. Expression and localization of Fas ligand and Fas during atresia in porcine ovarian follicles. *J Reprod Dev* (in submission).
68. **Matsuda-Minehata F, Goto Y, Inoue N, Manabe N**. Changes in expression of anti-apoptotic protein, cFLIP, in granulosa cell during follicular atresia in porcine ovaries. *Mol Reprod Dev* 2005; 72: 145–151.
69. **Nishi Y, Yanase T, Mu Y, Oba K, Ichino I, Saito M, Nomura M, Mukasa C, Okabe T, Goto K, Takayanagi R, Kashimura Y, Haji M, Nawata H**. Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor. *Endocrinology* 2001; 142: 437–445.
70. **Chedrese PJ, Rodway MR, Swan CL, Gillio-Meina C**. Establishment of a stable steroidogenic porcine granulosa cell line. *J Mol Endocrinol* 1998; 20: 287–292.
71. **Matsuda-Minehata F, Goto Y, Inoue N, Sakamaki K, Chedrese PJ, Manabe N**. Anti-apoptotic activity of porcine cFLIP in ovarian granulosa cell lines. *Mol Reprod Dev* (in press).
72. **Ratts VS, Flaws JA, Kolp R, Sorenson CM, Tilly JL**. Ablation of bcl-2 gene expression decreases the numbers of oocytes and primordial follicles established in the post-natal female mouse gonad. *Endocrinology* 1995; 136: 3665–3668.
73. **Hsu SY, Lai RJ, Finegold M, Hsueh AJ**. Targeted overexpression of Bcl-2 in ovaries of transgenic mice leads to decreased follicle apoptosis, enhanced folliculogenesis, and increased germ cell tumorigenesis. *Endocrinology* 1996; 137: 4837–4843.
74. **Morita Y, Tilly JL**. Oocyte apoptosis: like sand through an hourglass. *Dev Biol* 1999; 213: 1–17.
75. **Perez GI, Robles R, Knudson CM, Flaws JA, Korsmeyer SJ, Tilly JL**. Prolongation of ovarian lifespan into advanced chronological age by Bax-deficiency. *Nat Genet* 1999; 21: 200–203.
76. **Kugu K, Ratts VS, Piquette GN, Tilly KI, Tao XJ, Martimbeau S, Aberdeen GW, Krajewski S, Reed JC, Pepe GJ, Albrecht ED, Tilly JL**. Analysis of apoptosis and expression of bcl-2 gene family members in the human and baboon ovary. *Cell Death Differ* 1998; 5: 67–76.
77. **Robles R, Tao XJ, Trbovich AM, Maravel DV, Nahum R, Perez GI, Tilly KI, Tilly JL**. Localization, regulation and possible consequences of apoptotic protease-activating factor-1 (Apaf-1) expression in granulosa cells of the mouse ovary. *Endocrinology*

1999; 140: 2641–2644.
78. **Matsui T, Manabe N, Goto Y, Inoue N, Nishihara S, Miyamoto H**. Expression and activity of Apaf1 and caspase-9 in granulosa cells during follicular atresia in pig ovaries. *Reproduction* 2003; 126: 113–120.
79. **Chun SY, Billig H, Tilly JL, Furuta I, Tsafriri A, Hsueh AJ**. Gonadotropin suppression of apoptosis in cultured preovulatory follicles: mediatory role of endogenous insulin-like growth factor I. *Endocrinology* 1994; 135: 1845–1853.
80. **Baker J, Hardy MP, Zhou J, Bondy C, Lupu F, Bellve AR, Efstratiadis A**. Effects of an Igf1 gene null mutation on mouse reproduction. *Mol Endocrinol* 1996; 10: 903–918.
81. **Zhou J, Kumar TR, Matzuk MM, Bondy C**. Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. *Mol Endocrinol* 1997; 11: 1924–1933.
82. **Guthrie HD, Garrett WM, Cooper BS**. Follicle-stimulating hormone and insulin-like growth factor-I attenuate apoptosis in cultured porcine granulosa cells. *Biol Reprod* 1998; 58: 390–396.
83. **Sirotkin AV, Makarevich AV**. Growth hormone can regulate functions of porcine ovarian granulosa cells through the cAMP/protein kinase A system. *Anim Reprod Sci* 2002; 70: 111–126.
84. **Mao J, Smith MF, Rucker EB, Wu GM, McCauley TC, Cantley TC, Prather RS, Didion BA, Day BN**. Effect of epidermal growth factor and insulin-like growth factor I on porcine preantral follicular growth, antrum formation, and stimulation of granulosal cell proliferation and suppression of apoptosis in vitro. *J Anim Sci* 2004; 82: 1967–1975.
85. **Sun GW, Kobayashi H, Suzuki M, Kanayama N, Terao T**. Follicle-stimulating hormone and insulin-like growth factor I synergistically induce up-regulation of cartilage link protein (Crtl1) via activation of phosphatidylinositol-dependent kinase/Akt in rat granulosa cells. *Endocrinology* 2003; 144: 793–801.
86. **Hu CL, Cowan RG, Harman RM, Quirk SM**. Cell cycle progression and activation of Akt kinase are required for insulin-like growth factor I-mediated suppression of apoptosis in granulosa cells. *Mol Endocrinol* 2004; 18: 326–338.
87. **Quirk SM, Cowan RG, Harman RM, Hu CL, Porter DA**. Ovarian follicular growth and atresia: the relationship between cell proliferation and survival. *J Anim Sci* 2004; 82 E-Suppl: E40–52.
88. **Richards JS, Sharma SC, Falender AE, Lo YH**. Expression of FKHR, FKHRL1, and AFX genes in the rodent ovary: evidence for regulation by IGF-I, estrogen, and the gonadotropins. *Mol Endocrinol* 2002; 16: 580–599.
89. **Cunningham MA, Zhu Q, Unterman TG, Hammond JM**. Follicle-stimulating hormone promotes nuclear exclusion of the forkhead transcription factor FoxO1a via phosphatidylinositol 3-kinase in porcine granulosa cells. *Endocrinology* 2003; 144: 5585–5594.
90. **Park Y, Maizels ET, Feiger ZJ, Alam H, Peters CA, Woodruff TK, Unterman TG, Lee EJ, Jameson JL, Hunzicker-Dunn M**. Induction of cyclin D2 in rat granulosa cells requires FSH-dependent relief from FOXO1 repression coupled with positive signals from Smad. *J Biol Chem* 2005; 280: 9135–9148.
91. **Castrillon DH, Miao L, Kollipara R, Horner JW, DePinho RA**. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science* 2003; 301: 215–218.
92. **Hosaka T, Biggs WH 3rd, Tieu D, Boyer AD, Varki NM, Cavenee WK, Arden KC**. Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. *Proc Natl Acad Sci USA* 2004; 101: 2975–2980.
93. **Tilly JL, Billig H, Kowalski KI, Hsueh AJ**. 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 kinase-dependent mechanism. *Mol Endocrinol* 1992; 6: 1942–1950.
94. **Lynch K, Fernandez G, Pappalardo A, Peluso JJ**. Basic fibroblast growth factor inhibits apoptosis of spontaneously immortalized granulosa cells by regulating intracellular free calcium levels through a protein kinase C delta-dependent pathway. *Endocrinology* 2000; 141: 4209–4217.
95. **Chun SY, Eisenhauer KM, Kubo M, Hsueh AJ**. Interleukin-1β suppresses apoptosis in rat ovarian follicles by increasing nitric oxide production. *Endocrinology* 1995; 136: 3120–3127.