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

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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

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
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...
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 (cFLIPS) and a long form (cFLIP L). The structure of cFLIPS 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 cFLIPS 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 inhibit via granulosa cells. As a feedback mechanism, the inhibit 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 inhibit increased the number of ovulations in sows [43, 44], and injection of inhibit into the ovarian bursa of immature rats increased the number of medium-sized antral follicles [45], indicating that inhibit 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-1 (IGF-1), interleukin-1β (IL-1β), epidermal growth factor (EGF), Bcl-2, Bcl-XL, etc.] and atretogenic factors (inhibit, Bax, FasL, TNF-α, caspase, etc.) [48].

**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
the possible role of TRAIL in contrast to Fas 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.

![Working hypothesis on granulosa cell survival in healthy follicles (A) and atretic follicles (B).](image)

(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.
apoptosis, while almost all cells transfected with empty vector died, indicating the anti-apoptotic activity of pcFLIP in granulosa cells. When both cFLIP\textsubscript{S} and cFLIP\textsubscript{L}, or cFLIP\textsubscript{L} only, were suppressed by small interfering RNA (siRNA), the viability of the JC-410 cells decreased 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\textsubscript{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-1\textbeta 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-1\textbeta 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.

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