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Apoptosis in ovary

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TABLE OF CONTENTS

1. ABSTRACT

Folliculogenesis is a complex process involving dramatic morphological and functional changes in granulosa and theca cells. This process is sequential and dictated specifically by tightly regulated response to endocrine hormones and intra-ovarian regulators. In mammalian ovaries, only a few number of presented follicles in a fetal ovary can reach ovulatory status during follicular development; more than 99% of the follicles in the ovary undergo a degenerative process known as "atresia" induced by apotosis. It is characterized by distinct biochemical and morphological changes such as DNA fragmentation, plasma membrane blebbing and cell volume shrinkage. Apoptosis in ovary is regulated by a number of endocrine, locally produced intracellular mediators in a stage-specific and time-dependent manner. New knowledge of hormones and cell factors which regulate granulosa cell or oocyte apoptosis and their possible signaling pathways underlying intracellular events has made important contributions in advancing our understanding mechanism of follicular atresia.

2. INTRODUCTION

Apoptosis, also named programmed cell death, is a necessary process in a living organism to maintain proper development, and eliminate cell damage or excess (1, 2). In embryogenesis, for example, the fetus to form fingers and toes is needed by removing the tissue between them. It is also necessary for body to destroy cells that threatening integrity of organism (3). Over a century ago, there was no "term" for "apoptosis" or "programmed cell death". The earliest description of the physiological cell death recognized as distinct from pathological tissue destruction was derived from morphological evaluations (2). Later, a series of criteria were set up to identify the programmed cell death (4).

Apoptosis is an active form of programmed cell death, which is characterized by distinct biochemical and morphological changes, such as DNA fragmentation, plasma membrane blebbing and cell volume shrinkage. The characteristic structural and molecular events of apoptosis distinguish from necrosis, which is a group of cells die at

same time (4). Apoptosis always occurs in a single cell surrounded by viable cells. In multicellular organisms physiologically removing cells by apoptosis is a mandatory process in maintenance of homeostasis of individual. Apoptosis occurs in embryonegesis, embryo development and in various adult tissues, including reproductive tracts.

Generally it is thought there are two pathways for regulation of cell apoptosis: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (5, 6). In death receptor pathway, apoptosis is triggered by the binding of cell death receptors (Fas, TNFR etc.) located in the plasmic membrane with their complementary death activator (FasL, TNF etc.). The binding of death receptor and activator induces receptor accumulation and formation of a death-inducing signal complex (DISC). This complex recruits and activates a number of apoptotic executors, such as procaspase-8 (7-10). Activated caspases-8 also can cleave Bid, increasing its pro-death activity and translating it to mitochondria, where it causes the mitochondrial cytochrome *c* release. Both of these two pathways occur in gonads and uterus.

Apoptosis is an essential physiological process, by which tissues function normally. A balance of cell proliferation, differentiation and apoptosis plays an important role in a healthy organ, and any imbalance of the processes can lead to organ dysfunction and developmental abnormalities. Apoptosis in ovary is regulated by a number of endocrine, locally produced mediators. However, the signaling pathways underlying the intracellular events induced by these regulators are not clear. This chapter was focused on cell apoptosis in ovary by reviewing available data published in the recent literature, including our recent publications in the fields. The review would also address new knowledge of hormones and cell factors as well as their possible signaling pathways underlying the intracellular events, which regulate somatic and germ cell apoptosis in the gonad.

3. APOPTOSIS DURING FOLLICULAR DEVELOPMENT

3.1 Follicular development

During embryogenesis, primordial germ cells migrate from yolk sac through dorsal mesentery of hindgut to genital ridge, and the somatic cells derive from mesenchyme of genital ridge. Both of germ cells and somatic cells proliferate till each germ cell is enclosed by one layer somatic cells, named follicular epithelial cells to form primordial follicles. After mitosis occurred in somatic cells, the germ cells undergo first meiotic division, called primary oocytes. The primary oocytes become arrested in the diplotene stage of meiosis, until the primordial follicles start to grow and finally reach ovulatory stage.

Folliculogenesis is a complex process involving dramatic morphological and functional changes in granulosa and theca cells. This process is sequential and dictated by specifically, tightly regulated response to endocrine hormones and intraovarian regulators. They control follicular development by determining which of the growing follicles continue to develop and differentiate, and which become atretic. Mammalian ovaries contain thousands of thousand primordial follicles that are the only source of gametes during the entire reproductive life. However, there is study provides evidence that challenges the validity of the belief, the results showed bone marrow and peripheral blood as potential sources of female germ cells, that could sustain oocyte production in adulthood (11, 12). Primordial follicle consists of an oocyte surrounded by a single layer of flattened pre-granulosa cells. The primordial follicles may survive more than 50 years in woman ovary. Once a group of primordial follicles begin to grow, they will develop and differentiate either into dominant follicle (s) and ovulate, or undergo atresia at various stages of development. During onset of primordial follicle growth, flattened pre-granulosa cells become cuboidal and begin to proliferate. The enclosed oocyte begins to grow at the same time (13, 14). It is interesting to note why and how some primordial follicles are capable of starting to grow while their neighbor sisters remain quiescent. The signal (s) for selection of primordial follicle growth is not clearly known.

Growth of granulosa cells in the follicle is a key process in initiation and development of primordial follicle. The early growth stage of primary follicles (with mono-layer cuboidal granulosa cells) and secondary follicles (with stratified granulosa cells but without antrum) is characterized by a dramatic increase in proliferation of granulosa cells, identified in the rapid increase in number and size. Subsequently, granulosa cells separate from each other resulting in formation of follicular antrum, which is called antral follicle. Meanwhile, meiosis restarts in secondary oocyte, germinal vesicle (GV) disappearance, called GV breakdown (GVBD), and the first polar body divides. At last, the selected follicle burst, and the oocyte is ovulated, the rest of follicular cells further differentiate into a new endocrine organ, called corpus luteum (15).

3.2 Follicular atresia

In newly formed embryonic ovary, the germ cells leave mitotic cycle, and begin with meiotic divisin, the meiotic division of the oocytes become arrested in the first prophase (16). During both mitosis and meiosis, large numbers of germ cells are culled from the ovary for as yet unknown reasons, resulting in less than one-third of the total number of potential germ cells being endowed in the ovary within primordial follicles shortly after birth (17, 18). In human fetal ovaries, the maximum number of germ cells observed in 5 month of pregnancy is about 6.8×10^6. At birth, the number of germ cells in the primordial follicles has decreased markedly to less than 20% of the maximum number, due to apoptosis of germ cells occurring before formation of ovarian follicles (19). Detailed analyses of germ cell degeneration in rodent and human fetal ovary suggest that there are several discrete waves of germ cell loss occurring, such as attrition of dividing oogonia, degeneration of pachytene stage oocytes, and loss of diplotene stage oocytes (17-19).

Although male mammals generally retain germline stem cells for spermatogenesis in testis

throughout their adult life, oocyte production in most of female mammals is believed to cease before birth (16, 18, 20-22). The central concept of reproductive biology "basic biological doctrine that during the life of the individual there neither is nor can be any increase in the number of primary oocytes beyond those originally laid down when the ovary was formed" (23). But the belief of the concept has been challenged by Tilly *et al*, based on the data of rates of oocyte degeneration and clearance, as well as the chemical 9,10-dimethylbenz (α) anthracene (DMBA) induced synchronization of atresia in mouse ovary. They have demonstrated that in addition to existence of proliferative germ cells that sustain oocyte and follicle production in the postnatal mammalian ovary, the transgenic female mouse bone marrow may be also a potential source of germ cells to sustain oocyte production in adulthood (11, 12). However there is no evidence to determine whether those oocytes can fertilize normally and develop into viable offspring. Most ovarian follicles (>99.9%) present at birth never reach ovulatory status, but undergo atresia at various time point along this extended developmental pathway.

Apoptotic process follows a particular pattern during different phases: in fetal ovaries, most of apoptotic activity was detected in germ cells; while in adult quiescent cortical follicles apoptosis was occurred originating from both oocyte and granulosa cells. It has been demonstrated that it is the oocyte which initiates the apoptotic process and induces the follicular atresia. The process always begins from the oocyte and then extends to the surrounding follicular cells leading to the growing follicle atresia. (24). Thus, it seems that the apoptotic signals can communicate from a single cell to all over the other cell types inside the follicle. Finally, the whole follicular structure has become atretic, while the surrounding stromal cells remain viable.

It is interesting that although in the growing follicles, follicular atresia is mainly induced by granulosa cells, there species-specific difference in the apoptotic process was observed. The initiation area of granulosa cell apoptosis is different among species, it is therefore suggested that different local mechanisms to regulate apoptotic process may be present (25-27). For example, at the earliest stage of follicular atresia, the apoptotic granulosa cells are randomly scattered in ovaries of rodents; while in bovine ovary, apoptotic granulosa cells are located on outer surface of follicular wall; and in porcine ovary, apoptotic granulosa cells are located on inner surface of granulosa layer. Moreover, during early to middle stages of follicular atresia, there is no apoptotic cells observed in the theca external layer, although detachment and degeneration of granulosa layer, fragmentation of basement membrane, and the apoptotic endocrine cells in theca internal layer were observed.

4. HORMONAL REGULATION OF OVARIAN CELL APOPTOSIS

Physiological death of a cell is usually under control of multiple extracellular factors, and the balance of survival and apoptotic signals determines a cell fate. Follicular cell apoptosis is regulated by multiple hormones, including pituitary hormones as well as local growth factors, cytokines, and steroids (28, 29). Diverse hormones and growth factors can act as survival factors to inhibit apoptosis, or as apoptotic factors to induce cell demise, such as Fas ligand, tumor necrosis factor (TNF)-α, interleukin-6, and gonadotropin-releasing hormone (GnRH) (30-35), through endocrine, paracrine and autocrine mechanisms. The factors that regulate apoptosis in diverse tissues appear to be tissue-specific. In ovary, the action of these factors is dependent on the stage of follicular development as shown in Figure 1.

4.1 Primordial follicles

In primordial follicles, oocyte apoptosis is likely responsible for follicular degeneration. FSH is important for follicular development, however, FSH is unlikely to exert a direct action on primordial follicles because FSH receptors have not yet developed at this stage (36, 37). In fact, follicles do not express functional FSH receptors until the secondary stage of follicular development (36-38). Stem cell factor (SCF) is important for survival of primordial follicles in fetal as well as postnatal ovaries by preventing oocytes from apoptosis (39, 40). Another oocyte-derived factor which is important for survival of small follicles is growth and differentiation factor 9 (GDF-9). In mice lacking GDF-9 follicles do not develop beyond the primary or early secondary stage (41). Just recently we have demonstrated that androgen receptor (AR) and Foxo3a was expressed in 2 day-old mouse oocyte, treatment of the cultured ovaries with testosterone induced the ovarian Foxo3a phosphorylation and primordial follicular growth and development via PI3-K/Akt signal pathway. In contrast, the oocyte GDF9 was down-regulated by the androgen at late stage of culture via the same signal pathway. Therefore we suggested that intra-ovarian hyperandrogenism might be the main culprit for excessively growing follicle by inducing the Foxo3a exclusion from oocyte nucleoli and for follicular arrest by directly down-regulating GDF9 expression.

4.2 Preantral follicles

It has been reported that FSH is important for development of preantral follicles *in vivo*, and FSH can also enhance expression of steroidogenic enzymes (43, 44). Decrease of circulating gonadotrophins through hypophysectomy (45) or blockade of luteinizing hormone/follicle stimulating hormone (LH/FSH) surge (46) lead to massive atresia of pre-ovulatory follicles on the day of pro-oestrus. The hormonal regulation of apoptosis in granulosa cells during follicular atresia appears very complex, and probably involves a classic cell crosstalk of oocyte – granulosa cells - theca-interstitial cells, as well as interaction among the cells at different stage of follicular development (47-49). For example, when the preantral follicles isolated from 12-14 day old rats were cultured in serum-free medium, a spontaneous onset of apoptosis was observed in the granulosa cells, similar to that in preovulatory follicles. FSH, as well as its downstream mediator cAMP could not inhibit apoptosis of the granulosa cells in the cultured preantral follicles (50). This finding suggests that the action of gonadotrophins on

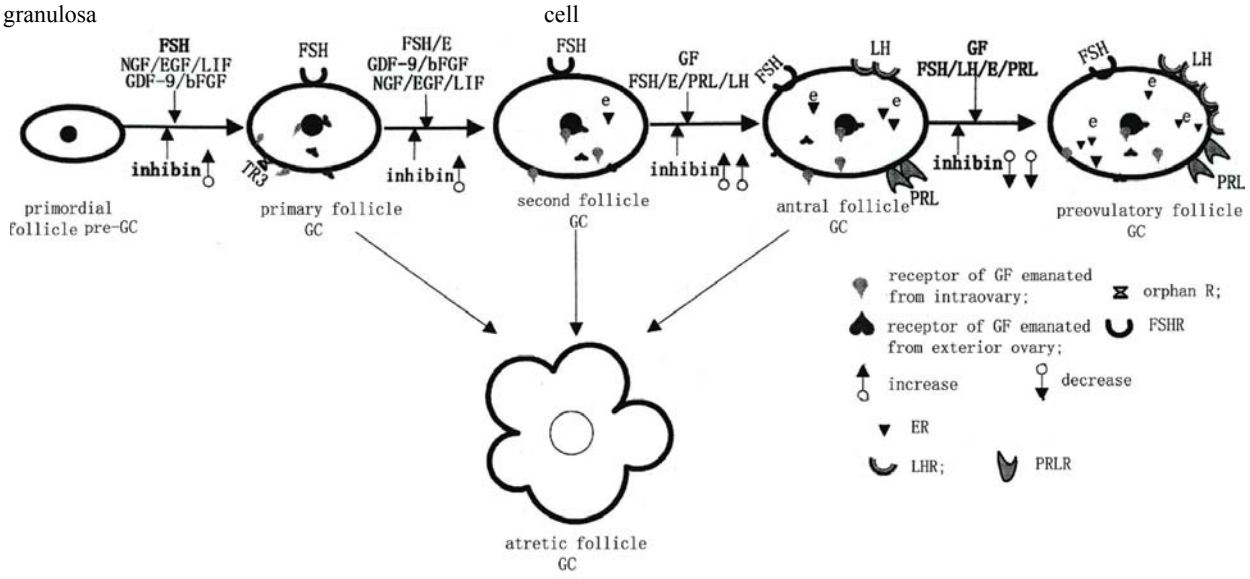

Figure 1. Progress of granulosa cell growth, differentiation and atresia. From the activation of the primordial follicle growth to selection of secondary follicle, growth factors appear to exert important effects on the growth and differentiation of granulosa cell (GC). Many factors, such as NGF, EGF, and GDF-9/bFGF as well are found to stimulate the initiation of primordial follicles. FSH receptor (FSHR) has been found in granulosa cells at primary stage, however, preantral follicle development is regulated predominantly by factors produced locally within ovary or follicle. FSH is not a necessary survival factor in the early developing preantral follicles. Progression of the antral follicle stage is the most critical stage of follicle development *in vivo*.GCs at this stage have acquired functional FSH receptors□their proliferation and differentiation are driven mainly by FSH and LH (at the preovulatory stage), and modulated by other extrinsic and locally produced factors which may have both stimulatory and inhibitory action on GC, such as inhibin. Inhibin was first identified in ovarian follicular fluid and demonstrated its ability to suppress FSH secretion. Apoptosis occurs at each stage of follicular development in the control of the indicated factors. Reproduced with permission from (154).

apoptosis may be mediated via other gonadotrophin – sensitive cells, such as cells in follicles at a later stage of development, rather than directly on the preantral follicles. Besides gonadotrophins, locally produced survival factors may also play important roles in regulation of follicular atresia. Sex steroids have been demonstrated to be essential important ovarian factors for follicle development (51). In human, ovine or porcine ovaries estradiol production by atretic follicles was lower, while androgen production was higher (52-54). However, in rat and hamster ovaries, the production of both estrogen and androgen decreased in atretic follicles (46, 55-57). In general, changes in steroidogenesis can be observed prior to morphological signs of atresia (46, 55, 58). Liu *et al* have demonstrated that endogenously-produced estrogen in the developing follicles synergistically with FSH can enhance the aromatase activity and differentiation of LH receptors□and is essential for follicular differentiation and dominate follicle formation. Decrease in endogenously estrogen in the follicle may lead to its atresia (59, 60).

In-situ analysis of DNA fragmentation on histological sections of ovaries has demonstrated that apoptosis induced by estrogen withdrawal in hypophysectomized rats is confined to the granulosa cells in early antral and pre-antral follicles, but no increase in DNA breakdown in primordial and primary follicles was demonstrated (51). ERαβ double knock-out mice are infertile because of follicular arrest (61, 62). Nevertheless, early follicular growth and development can also occur in these mice, even though mature Graafian follicles do not form.

In contrast to estrogen, which inhibits granulosa cell apoptosis, androgen promotes the cell apoptosis (28). *In vivo*, treatment with androgen causes a dose- and time-dependent decrease in ovarian weight (63, 64) and an increase in morphological signs of atresia in estrogen – treated hypophysectomized rats (64).

Other locally produced growth factors, including keratinocyte growth factor (KGF), fibroblast growth factor (bFGF) are also important for survival of preantral follicles. KGF, a member of FGF family which produced by thecal cells and receptors of that are present in granulosa cells, suppresses apoptosis in cultured rat preantral follicles (65). Similar effect of FGF on apoptosis has also observed in cultured preantral follicles (65).

4.3 Early antral follicles

In human and rodent, the early antral stage of follicle development is the most critical stage. At this stage a functional FSH receptor is expressed in the granulosa cells, and follicle survival becomes mainly dependent on FSH stimulation (13). FSH is able to suppress apoptosis by up to 60%, but its action was partially reversed by insulin

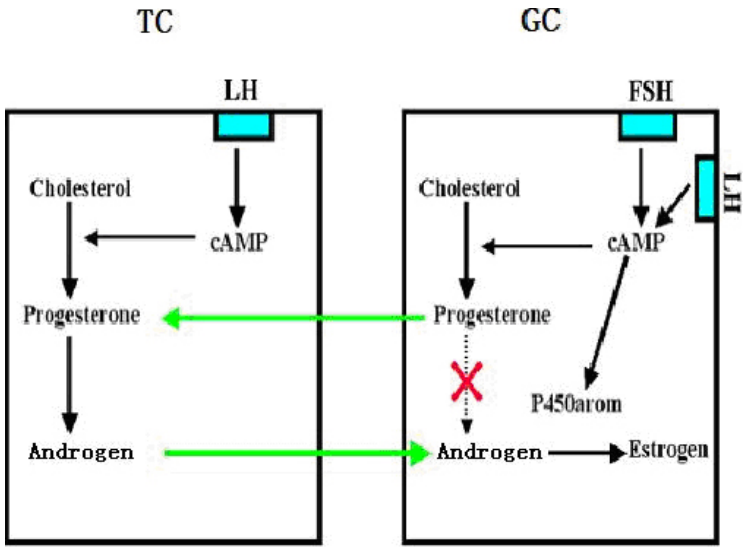

Figure 2. A representative diagram showing involvement of interaction between granulosa cell and theca cell in estrogen production in the follicle. Under the regulation of LH, the theca cell (TC) synthesizes androgen, but it can be not converted into the estrogen because of lacking P450arom in TC; while the granulosa cell (GC) under the action of FSH (also by LH at the late stage) synthesizes progesterone, the later, however can not be converted further into androgen because of lacking the necessary converting enzymes in GC, although GC has the P450arom, but can not synthesizes estrogen by itself. Progesterone produced by GC can be used by TC to convert androgen, while GC is capable of using the androgen produced by TC to convert into estrogen. The interaction of TC and GC in the follicle is a prerequisite for estrogen biosynthesis in the ovary and may produce the highest level of estrogen in the PMSG-treated follicles. Drawing reproduced with permission from (90).

growth factor (IGF) binding protein IGFBP-3, suggesting that some of the physiological effects of FSH may be mediated by IGF (66). Local production of IGF-I plays an important intra-ovarian role in augmentation of gonadotrophin stimulation of follicle differentiation. (67, 68). IGF and its binding protein (IGFBP) are important for oocyte maturity, and granulosa cells differentiation during follicle development. Gene expression for IGF-binding protein in granulosa cells definitely differed between normal women and women with polycystic ovary syndrome (69-72). IGFBP-4 and -5 are produced by rat granulosa cells (73, 74). FSH treatment increases IGF-I production (75, 76), but decreases IGFBP secretion in ovaries (77). High concentrations of IGFBP have been detected in atretic human follicles of both normal and polycystic ovarian syndrome patients (78, 79). *In situ* mRNA analysis has further demonstrated presence of IGFBP in atretic but not in healthy follicles (74). IGF-I, as well as FSH prevent spontaneous onset of apoptosis in cultured follicles (47), however, they can not prevent apoptosis in isolated granulosa cells, in spite of presence of their receptors on the granulosa cells (80), indicating that theca cells may be important for mediating the suppressive effect of IGF-I and gonadotrophins on apoptosis.

The mechanisms controlling the follicular growth involve the interaction between local growth factors which are expressed throughout development and extra-follicular factors. A large number of follicular growth factors, such as member of bone morphogenetic family, BMP-15, epidermal growth factor (EGF) and growth differentiation factor-9 (GDF-9) control the initiation of follicular growth and early preantral development. During antral follicle development, the oocyte secretes factors that stimulate granulosa cell proliferation and differentiation, modulate apoptosis and suppress progesterone production, thereby preventing premature luteinisation (81, 82). In the development competence of *in vitro*-matured (IVM) cumulus oocyte complexes (COCs), epidermal growth factor (EGF) has been proved functionally mimicked the action of FSH and could completely replace FSH for nuclear maturation, specific inhibition of EGF receptor (EGFR) inhibited both EGF- and FSH-induced meiotic resumption (83-87). Besides of EGF, growth hormone (GH), IGFs and IGFBPs also play an important role in preantral follicle growth through their binding with GH receptor, which are located both in the oocyte and follicular somatic tissues. *In vitro* studies and knockout experiments shows GH stimulates the development of small antral follicles to gonadotrophin-dependent stages, as well as maturation of oocytes. In antral follicles, IGFs stimulate granulose cell proliferation and steroidogenesis in most mammals (88, 89).

4.4 Preovulatory follicles

At the preovulatory stage, both granulosa and theca cells in the follicle express LH receptors and are able to respond to impending LH surge. It has been reported that FSH and LH both suppressed the degree of apoptosis in isolated preovulatory rat follicles (47). At this stage, interaction of granulosa cells and theca cells produces the highest estrogen in the follicle, as shown in Figure 2**,** that may be important for preventing the selected dominate follicle atresia and going to ovulation (90).

Endogenous IGF-1 also partially mediates suppression of apoptosis by gonadotrophins. LH receptor stimulation results in an increase in IGF-1 mRNA content in cultured preovulatory follicles, while IGFBP-3 results in a dose-dependent decrease in the apoptosis suppressive effect by LH receptor stimulation (47). Besides of IGF-1, cytokine IL-1β mediates part of apoptosis suppressive effect of gonadotrophins in rats, while IL-1β receptor antagonist partially decreases effect of gonadotrophins (91). Insulin is another survival factor for cultured rat preovulatory follicles. Although insulin has no effect on isolated preovulatory rat granulosa cells, it can decrease sensitivity of cultured follicles to apoptosis, suggesting involvement of other ovarian cells (80). EGF, bFGF, and GH suppress apoptosis effectively in follicles at preovulatory stage.

4.5 Periovulatory follicles

Follicles that survive to periovulatory stage are dependent on endogenous LH surge. After LH surge, follicles are less susceptible to atresia than those are at earlier stages (92). Inhibition of LH surge by hypophysectomy or pentobarbital treatment causes follicles to degenerate (93, 46). The suppression of LH to apoptosis is partly mediated by endogenous production of pituitary adenylate cyclase-activating polypeptide (PACAP) (94).

Shortly after LH surge, expression of nuclear progesterone receptor is induced in both rat and human granulosa cells, which coincides with apoptosis suppressive effects of progesterone. In rat, expression of progesterone receptors is transient, while in human, it is prolonged (95, 96). Progesterone functions as a regulator of apoptosis via its nuclear receptor at periovulatory follicles (92). Mice lacking both isoforms of the progesterone receptor (A and B) are anovulatory, indicating that progesterone has a direct effect in ovary (97, 98). Progesterone regulates expression of the genes, such as PACAP, and its receptor PAC1 (99, 100). In immature and preovulatory stage (before LH surge), progesterone attributes an apoptosis-inhibiting effect via a GABA receptor-like receptor, but that seems not related with apoptosis (92).

5. APOPTOSIS IN COUPUS LUTEUM

The corpus luteum (CL) is developed by extensive cellular reorganization and neovascularization of remnants of evacuated follicle following ovulation. CL is a transient endocrine organ that secretes progesterone to support early pregnancy. If implantation is unsuccessful, luteolysis is initiated. In both rodent and primate, development of CL is a rapid process with very high cellular turnover (101-103). A CL is usually developed within hours in rat and mouse, and within days in monkey and human. A mature CL receives the greatest blood supply per unit tissue in the whole body (103). However, if the implantation is unsuccessful, the functional phase of the CL is terminated and luteolysis is initiated. Associated with these repetitive cycles of luteal development and regression is an extensive connective tissue remodeling and extracellular matrix degradation (104, 105).

It has been reported that CL function is regulated by various bioactive substances, such as gonadotropins, steroids, and growth factors. CL regression can be initiated by the release of PGF2α from uterus. PGF2α inhibits the gonadotropin-stimulated CL progesterone production, after the initial decrease in the steroidogenesis, more chronic effects of PGF2α take place, including loss of gonadotropin receptors and disruption of the cytoskeleton, and preventing progesterone secretion, eventually followed by morphological changes in the steroidogenic cells, and loss in both size and weight of CL (106). Another important factor initiating CL regression is prolactin secretion in the estrous cycles (107). Using a chemical drug to block prolactin secretion, corpora lutea are increased by weight (108). Furthermore, chemical blockade of the proestrous prolactin surge diminishes apoptosis in the regressing corpora lutea (109).

Luteal regression is a complex process that involves two phases. The first phase, named functional regression which means the functional ability of corpus luteum to sustain pregnancy lost at this stage, is defined as termination of secretion of appreciable quantities of progesterone (110) and occurs during the 4- to 5-day estrous cycle. The second phase, called structural luteolysis, is defined as the complete morphological regression of corpus luteum mainly by cell apoptosis. A great decrease in weight and size occurs at this stage. The latter process is executed long after the initial decline in progesterone secretion and corpus lutea may remain in the ovary throughout several estrous cycles before their complete dissolution (111, 112). The main mechanism involved in reduction in size and weight of corpus luteum is removal of luteal cells by apoptosis and subsequent phagocytosis (113, 114).

Our serious experiments in monkey, rat and mouse have demonstrated involvement of matrix remodeling proteases in the processes of tissue remodeling during CL formation and luteolysis. Matrix remodeling proteases includes plasminogen activator (PA) (115, 116) and matrix metalloproteinase (117-123) system. Our experiment results suggest that coordinated expression of tissue type plasminogen activator (tPA) and its inhibitor type-1 (PAI-1) in corpus luteum at late stage of CL development in primate, rat and mouse induces CL regression, leading to luteal cell apoptosis (115, 116, 124). In addition to the PA system, recent evidence suggests that the luteal tissue remodeling is also regulated by MMP/TIMP system (117). The TIMPs have been reported to stimulate cell growth, impact angiogenesis, and induce cell apoptosis (125-127). Recently we demonstrated that coordinated expression of MMP-2, -14 and TIMP-1, -3 may have a potential role in the CL formation and the function, while the interaction of MMP-2, -9, -14 and TIMP-1, -2, -3 might also play a role in CL regression at the late stage of CL development in the primate (127).

At early and late stages of CL development an extensive tissue remodel and cell apoptosis occur. Using VEGF and its receptors as well as StAR as the marker molecules of CL function, we have designed experiments

to look at the possible effect of cytokines and RU486 on CL regression and apoptosis (128-133). As compared to the control, a single administration of RU486 significantly increased VEGF expression in the CL during early pregnancy in monkey. However, twice administration of RU486 significantly declined the single-RU486-induced VEGF expression (p=0.005), the mechanism, however is not known.

IFN-γ can inhibit progesterone production of luteal cells (128, 129, 132, 133) and induce luteal cell apoptosis in varies species including human (134, 135). After treatment of monkey with IFN-γ, as compared to the control, the sharp endothelial cells in the CL altered into round. However, unlike TNF-α treatment, the VEGF levels did not increase after administration of IFN-γ. These results suggest that endothelial cells might be going to degenerate, IFN-γ may induce angiolysis in the primate CL even at the early state of CL development. Thus, IFN-γ could promote luteal degression by inducing the cell apoptosis in the primate.

Evidence in recent years has shown that caspase-3 exerts an important role in luteal cell apoptosis of bovine and cattle (136, 137). It has also reported that activation of protein kinase C (PKC) signal pathway and cAMP accumulation could protect bovine luteal cells from apoptosis by suppressing caspase-3 mRNA expression (138).

6. SIGNAL PATHWAYS OF THE FACTORS IN REGULATING APOPTOSIS IN OVARY

Selection of apoptosis or survival of granulosa cells and oocyte is a critical process in determining the fate of follicular development. In mammals, at least 60 different proteins and signaling molecules have been identified as constituents of intracellular framework that governs apoptosis. Although a number of endocrine and paracrine factors have been shown as survival or apoptosis-inducing factors in these cells *in vivo* or *in vitro* (139, 140), their molecular mechanisms and the underlying intracellular events are not completed illuminated. Several intracellular signaling pathways have been linked directly to promoting granulosa cell or oocyte survival, including pathways such as gonadotrophin- and vasoactive intestinal peptide (VIP)-induced cAMP formation (48, 141), mitogen-activated protein kinase (MAPK) (142), and phosphoinositol-3-kinase-Akt (143, 144). PI3K/Akt pathways play an important role in mediating anti-apoptotic action of SCF in oocytes of primordial follicles. Jin *et al* (39) demonstrated that the anti-apoptotic effect of SCF on oocytes was significantly inhibited by the PI3K inhibitor (Figure 3). Moreover, PI3K inhibitor could also revere the effect of SCF on the expression of Bcl-xL and Bax (Figure 4). MAPKs activation is a key event in many cellular processes, including proliferation, differentiation, and apoptosis (145). There are three main classes of MAPK: Erks, c-Jun amino-terminal kinases (JNKs), and P38 proteins (146-148). Erks are important mediator of many factors, such as FSH (149), SCF (150). Inhibition of Erks activity with PD98059 distinctly reduces FSH-induced DNA synthesis in immortalized granulosa cells and over-expressing a recombinant novel growth factor type 1 receptor for FSH. Study on granulosa cells isolated from equine chorionic gonadotropin-primed immature rats revealed that activities of Erks,MEK kinase and Raf-1 were reduced with a concomitant decrease in phosphorylation level of the proapoptotic factor, Bad, prior to onset of granulosa cells apoptosis (142). Another important signal molecule stimulated by FSH is cyclic AMP (cAMP), signaling via cAMP enhances resistance of hen granulosa cells to apoptosis. Blocking cellular phosphodiesterase activity in forskolin-stimulated primary granulosa cells by isobutylmethylxanthine, which maintains high level of intracellular cAMP, led to further enhancement of cell death (141, 48).

It has been reported that the regulated phosphorylation of Tyr residues is a major control mechanism for the processes as diverse as cell survival, proliferation, differentiation, and metabolism. The opposing activities of protein tyrosine kinases (PTKs) and PTPs accurately regulate protein phosphor-Tyr (pTyr) levels (151, 152). Several reports indicated that PTKs play important roles in regulating intracellular events of granulosa cells after stimulation with various factors (47, 66, 80, 153). For instance, EGF, IGF/insulin and bFGF prevent spontaneous onset of apoptosis in cultured granulosa cells by activating their respective tyrosine kinase receptors (47, 80). IL-1β acts through cytoplasmic PTKs, called Janus kinases (JAKs), that is an effective survival factor for preovulatory follicles *in vitro* (66). Other signal molecules, such as Ca^{2+}, protein kinase C, heat-shock proteins may also involve in transduction of factors regulating the apoptosis in ovary.

Using DNA 3'-terminal labeling, immunohistochemistry, *in situ* hybridization we comparatively examined the correlation expression of inhibin, LH receptor in granulosa cells, and the tPA activity in oocytes at the same section of the follicle. High level of tPA mRNA in oocytes was detected at early stages of follicular development, but tPA protein activity in the oocyte was not detected until the onset of meiosis maturation at late stage of follicular development triggered by the LH surge. The high level expression of inhibin in GC observed in the early stage follicles may play an essential role in preventing the tPA mRNA translation into its protein in the oocytes. Once inhibin expression decreases in the GC of developing follicles at early stage, the increasing tPA protein activity in the oocyte may induce certain morphological changes in the oocyte similar to GVBD, leading to the oocyte apoptosis and the follicle atresia in the under-developed follicles. Based on this finding, we have proposed a mechanism of follicular atresia originating from oocyte apoptosis (Figure 5) (154, 155)

6.1 Intracellular molecular mechanism of ovarian cell death

Although there are many different hormonal signals to regulate apoptosis in ovary, the intracellular cascade of events appears to share common features (Figure 6.). Bcl-2 system is important in regulation of

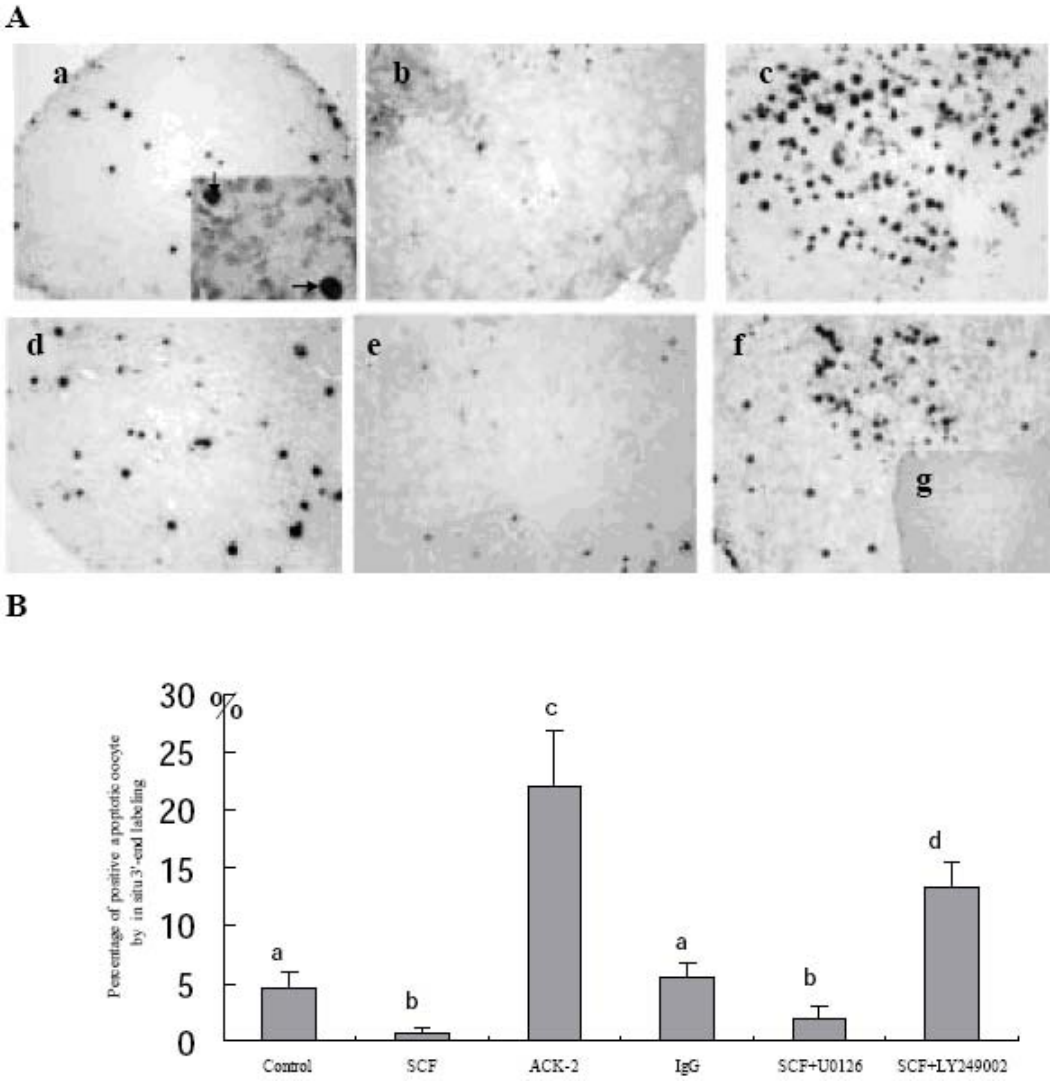

Figure 3. Anti-apoptotic action of SCF on oocytes in cultured ovaries. (A) Apoptotic nuclei are stained dark using the *in situ* 3'-end labeling technique. a□Cultured ovaries without any treatment. Inset is area of higher magnification showing apoptotic staining is localized in nuclei of oocytes (arrows). b) Ovaries treated with 100ng/ml SCF for 2 days. c) Ovaries treated with SCF plus c-kit antibody. d) Ovaries treated with SCF plus IgG. e). Ovaries treated with SCF plus MEK inhibitor U0126. f) Ovaries treated with SCF plus PI3K inhibitor LY294002. g) Ovaries without SCF treatment and incubated with normal serum IgG as a negative control. Magnification is 400× (magnification of inset is 1000 ×). (B).Statistical analysis of apoptotic oocytes in ovaries of different treatments. Vertical axis represents the percentage of apoptotic cells over total number of oocytes (mean±SEM, n=3) Statistical analysis was performed using ANOVA followed by the Student-New-Man-Keuls multirange test. Bars with different letters indicate statistically significant differences ($P<0.05$). Reproduced with permission from (39).

ovarian cell apoptosis *in vivo*. Bcl-2 is a proto-oncogene, which encodes a membrane-anchored intracellular protein to prevent apoptosis induced by various stimuli (156, 157). Expression of Bcl-2 has been detected in ovary of many species (158, 159). In the transgenic mice over-expression of Bcl-2 was detected in the ovary, while the follicular cell apoptosis was suppressed, and followed by enhancing folliculogenesis and an increased incidence of benign ovarian teratoma development, indicating that Bcl-2 associated regulatory system is operating in the ovary (160). In contrast, ablation of functional Bcl-2 through targeted disruption of the gene (gene 'knock-out') leads to significantly fewer oocytes and primordial follicles in the postnatal ovary (161). Another member of Bcl-2 gene family is Bax, a death-susceptibility gene. The protein of Bax was originally identified via its ability to non-covalently interact with Bcl-2 in cells (162). This interaction is thought to blunt Bcl-2 bioactivity and thus serve as proapoptotic member. With oocyte in-vitro maturation experiment, Bcl-2 mRNA expression is significantly higher in cumulus-oocyte-complexes (COC) cells associated with mature oocytes than those associated

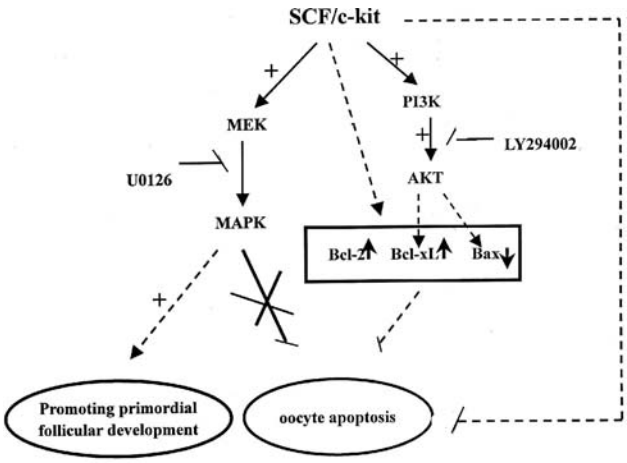

Figure 4. Schematic representation of potential signal pathways elicited by SCF in the oocytes of primordial follicles. SCF elicits an anti-apoptotic signal starting from its membrane receptor c-kit. The signal is cascaded downstream through PI3K/AKT proteins resulting in the changes in expression of the Bcl-2 family members Bcl-xL and Bax. The expression regulation of Bcl-2 by SCF might be through other pathway (s) that does not contain the PI3K/AKT module. MAPKs are activated by SCF, but unable to propagate the anti-apoptotic signal. They might affect other aspects of follicular development (see discussions). + and – are symbols for stimulating and inhibitory effects, respectively. Solid line arrows represent for direct interaction, while broken line arrows stands for an indirect action. Upward and downward arrows stand for up- and down- regulation of gene expression. Reproduced with permission from (40).

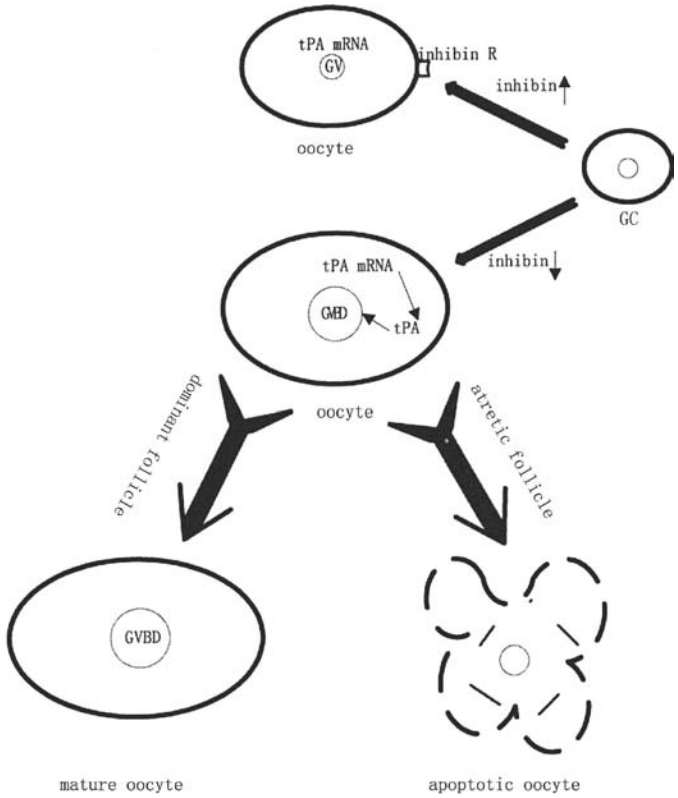

Figure 5. Schematic diagram showing inhibin produced by granulosa cells controls oocyte tPA mRNA translation. Inhibin originated from the granulosa cell (GC) inhibits the oocyte maturation by inhibiting tPA mRNA translation in the oocyte. Once inhibin expression in GC is decreased, the oocyte tPA mRNA starts to translate into its active protein, the subsequently increased tPA activity induces the oocyte GVBD in the dominant follicle leading to the oocyte maturation and ovulation; On the other hand, decreases in GC inhibin expression in developing follicle, the oocyte tPA mRNA is triggered to translate tPA protein which is capable of inducing its certain morphological changes similar to GVBD in the developing follicle, subsequently leading to the oocyte and / or the follicle apoptosis. Reproduced with permission from (153,154).

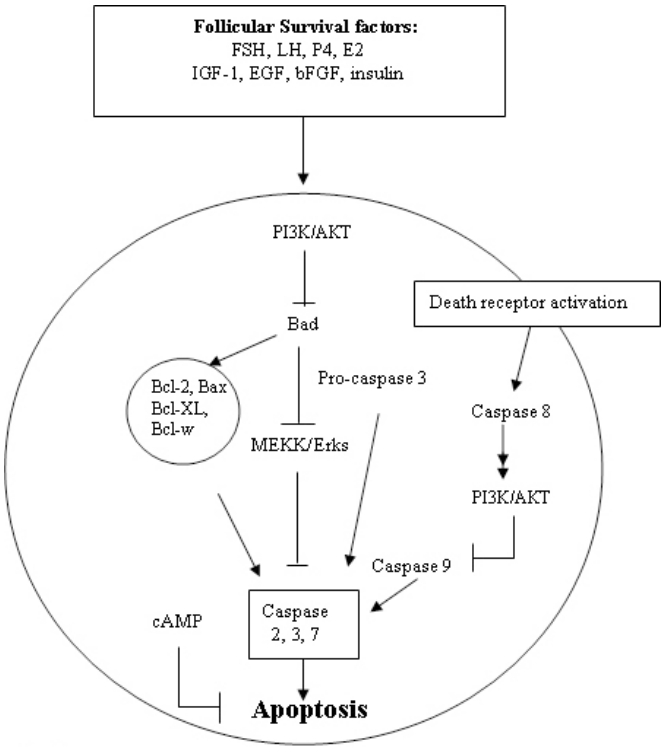

Figure 6. Schematic overview of apoptotic process in granulosa cells.A range of survival factors, for instance, FSH and LH, progesterone (P4), estrogen (E2), IGF-1, EGF, bFGF, insulin, and locally produced factors control death of the cell. Survival factors utilize a number of signal transduction molecules, such as PI3K/AKT, to regulate the expression of Bcl-2 family members. Another important intracellular cysteine proteases are caspases, which are linked both to the initial and final stages of apoptosis. Caspases include "initiator" factors, such as caspase-8, and -9, are directed by upstream apoptotic signals and downstream "effector" caspases, such as caspase-2, -3 and -7, are activated by initiators and function in the subsequent degradation of cellular components. Besides of PI3K/AKT pathway, MEKK/Erks and cAMP are also involved in the cellular apoptosis controlling in the ovary. Reproduced with permission from (39,40, 48, 141, 142).

with immature oocytes, and levels of Bax expression appear to be positively correlated with apoptosis in each of these cell lineages (163-165). Knudson and his colleagues (166) noted "a marked accumulation of unusual atretic follicles" containing "numerous atrophic granulosa cells that presumably failed to undergo apoptosis". Primordial oocytes within the ovaries of Bax null mice were completely resistant to apoptosis induced by exposure to a widely used chemotherapeutic drug *in vivo* (167). Similarly, granulosa cells within degenerating follicles of Bax-deficient mice also appear to be resistant to induction of apoptosis (166). A significant defect in primordial and primary follicle atresia rates was detected in Bax-deficient female mice, leading to a marked reduction in the incidence of postnatal oocyte death. Moreover, in aged Bax mutant females, defect in oocyte death leads to a dramatic prolongation of ovarian life span (168). These results support a fundamental role for Bax in mediating apoptosis in both oocyte and granulosa cells.

In addition to Bcl-2 and Bax, several other members of Bcl-2 gene family have been found expressed in ovary and play important role on oocyte survival (169, 170, 164), such as Bad, which acts as an important pro-apoptotic ligand by bridging upstream signaling proteins, 14-3-3 and P11, to the channel-forming anti-apoptotic Bcl-2 family proteins (171). In the ovary, Bad plays an important role in mediating communication from different upstream signal transduction pathways to the Bcl-2 regulated apoptotic decision step. Gonadotropins and other upstream survival factors, such as IGF-1 and insulin, activate Akt/PKB kinase to phosphorylate Bad to allow binding of 14-3-3 proteins, leading to dampening of Bad-induced cell killing (171-174). Bad phosphorylation has been suggested to be an important mechanism by which upstream survival factors suppress apoptosis.

7. CONCLUSION AND ESPETIVES

Apoptosis in ovary is a complex, but a regulated process, it plays important roles in reproduction under various physiological conditions. Dysregulation of cell apoptosis in the reproductive tract causes infertility.and reproductive diseases.

Apoptosis often begins before birth, and continuously throughout reproductive life. Balance of cell proliferation and apoptosis plays an important role in a healthy organ, any imbalance of these two processes can lead to organ dysfunction and developmental abnormalities.

To better understand mechanism of cell apoptosis can help to find ways to prevent its inappropriate occurrence and to improve reproductive health and give more helpful insight on treatment of reproductive diseases.

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