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Alternative pathways of ovarian apoptosis: death for life

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Abstract

Ovarian cell death is an essential process for the homeostasis of ovarian function in human and other mammalian species. It ensures the selection of the dominant follicle and the demise of excess follicles. In turn, this process minimizes the possibility of multiple embryo development during pregnancy and assures the development of few, but healthy embryos. Degeneration of the old corpora lutea in each estrus/menstrual cycle by programmed cell death is essential for maintaining the normal cyclicity of ovarian steroidogenesis. Although there are multiple pathways that can determine cell death or survival, crosstalk among endocrine, paracrine and autocrine factors, as well as among protooncogenes, tumor suppressor genes, survival genes and death genes, play an important role in determining the fate of ovarian somatic and germ cells. The establishment of immortalized rat and human steroidogenic granulosa cell lines and the investigation of pure populations of primary granulosa cells allows for systematic studies of the mechanisms that control steroidogenesis and apoptosis of granulosa cells. We have discovered that during initial stages of granulosa cell apoptosis progesterone production does not decrease. In contrast, we found that it is elevated for up to 24 hr following the onset of the apoptotic stimuli exerted by starvation, cAMP, p53 or tumor necrosis factor α stimulation, before total cell collapse. These observations raise the possibility for an alternative unique apoptotic pathway, one that does not involve mitochondrial cytochrome C release associated with the destruction of mitochondrial structure and steroidogenic function. Using mRNA from apoptotic cells and Affymetrix DNA microarray we discovered that Granzyme B, a protease that normally resides in T cytotoxic lymphocytes and natural killer cells of the immune system is expressed and activated in granulosa cells, thereby allowing the apoptotic signals to bypass mitochondrial signals for apoptosis, which can preserve their steroidogenic activity until complete cell destruction. This unique apoptotic pathway assures the cyclicity of estradiol and progesterone release in the estrus/menstrus cycle even during the initial stage of apoptosis.

Keywords: Granulosa cells; Granzyme B; p53; Actin cytoskeleton; cAMP; TNF-α

1. Introduction

Ovarian cell death is a crucial event in maintaining ovarian homeostasis in mammals. It ensures that in every estrus/menstrus cycle only one or very few follicle-enclosed oocytes will reach the stage of a Graafian follicle and will ovulate. This is essential in preventing multiple embryos during pregnancy. The rest of the follicles are gradually eliminated during the fertility period of the female. The apoptotic process of the old corpora lutea is essential for preserving the cyclicity and for ensuring the release of progesterone during the estrus/menstrus cycle (reviewed in [1–5]). There are several factors that may control apoptosis of granulosa cells. In each stage of the cycle about 50% of the large preantral and antral follicles are in the process of apoptotic death [6]. In most antral follicles apoptosis is initiated at inner layers of the membrana granulosa, bordering the follicle antrum (Fig. 1). Apoptosis is protected by the basement membrane which can sequester bFGF where both laminin, the main component of the basement membrane, and bFGF serve as survival factors [7,8]. Interestingly,

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Abbreviations: TNFα, tumor necrosis factor α; Cyt C, cytochrome C; bFGF, basic fibroblastic growth factor; EGF, epidermal growth factor; IGF, insulin-like growth factor; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; LH, luteinizing hormone; FSH, follicle stimulating hormone; FK, forskolin.

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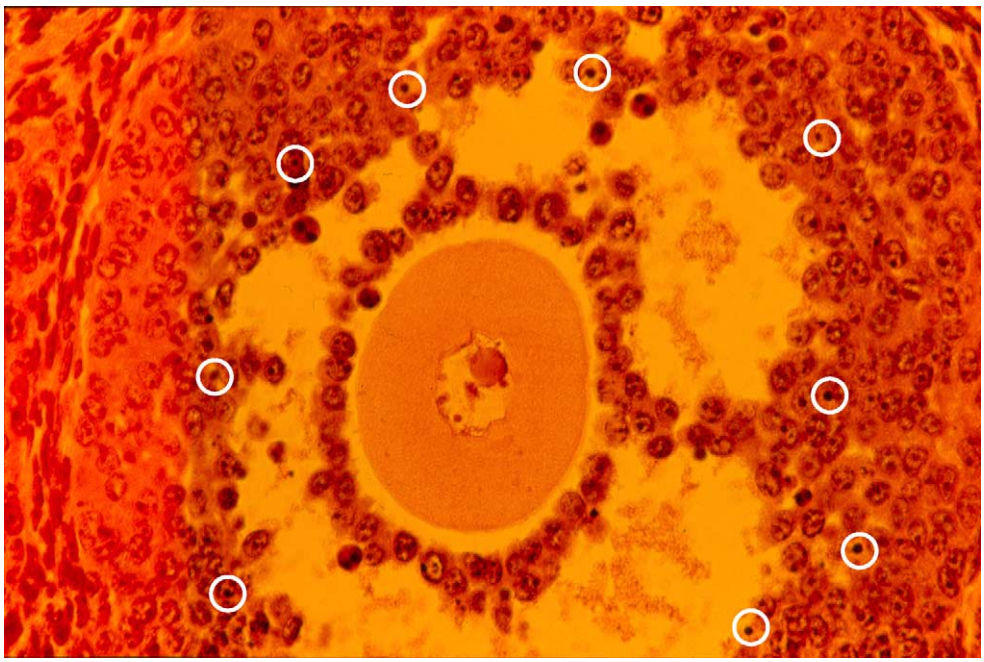

Fig. 1. Antral follicle of rat ovary. The oocyte in the center is sectioned at the level of the germinal vesicle and the nucleolus. Although the oocyte seems intact with no sign of apoptosis, high incidence of apoptosis appears in the inner layers of the granulosa cells bordering the antrum (white circles). Hematoxylin–eosine staining 1000×.

laminin protects against apoptosis without affecting steroidogenesis, while bFGF enhances formation of progesterone in preovulatory follicular cells [7].

Induction of apoptosis *in vivo* by hypophysectomy of female rats surprisingly elevates progesterone production dramatically in the ovary [9]. This may suggest that the initial steps of apoptosis enhance rather than block progesterone production. A similar phenomenon is found upon induction of apoptosis in highly luteinized granulosa cells [10–12] (Fig. 2) which raises the question: can steroidogenesis and apoptosis exist in the same cell? If the answer is positive, then the next question is how mitochondrial integrity, which is the prime target of apoptotic signals in numerous cell types, is preserved during the initial steps of apoptosis in ovarian steroidogenic cells?

2. The sensory world of the granulosa cell: control of steroidogenesis and apoptosis

Increasing evidence suggests that there are alternative apoptotic stimuli in the ovarian follicle as well as in cultured granulosa cells [3]. Some of the stimuli negate steroidogenesis as TNFα [13], while some of them enhance steroidogenesis as high levels of intracellular cAMP [10]. Stimuli for apoptosis or survival can be endocrine, paracrine and autocrine [3]. The main survival factors are gonadotropins, EGF, IGF, FGF, prolactin, laminin, leptin, glucocorticoids and estradiol (Fig. 3), while the main apoptotic signals are TNFα, high levels of cAMP and gonadotropin releasing hormone (GnRH). Moreover, some apoptotic signals can synergize with each other like high levels of cAMP and activation of p53 [3,14] and some survival factors such as steroid hormones and growth factors can negate apoptotic signals evoked by p53 and TNFα [3,13]. Interestingly, we recently discovered that gonadotropic hormones, LH/chorionic gonadotropin (CG) and FSH may exert their survival activity via activation of the MAP kinase cascade in primary and immortalized cell lines established in our laboratory [15,16]. This stimulation leads to rapid phosphorylation of ERK1 and ERK2 in both cell systems.

We conclude that crosstalk among different signals determines the fate of the ovarian follicle. Because of multiple signals, some of which are paracrine and/or autocrine, it is still not completely understood which are the critical factors that discriminate between follicles destined for elimination by apoptosis (the major population of follicles) and those follicles that will continue to develop to reach the final stage of Graafian preovulatory follicle.

3. Mechanism of action: relationship between steroidogenesis and apoptosis

Early studies of hypophysectomy of female rats demonstrate a dramatic increase in progesterone formation and release from the ovarian follicle with a progressive increase in atresia (apoptotic cells) which reach 20–30% of the total granulosa cell population within 48 hr [9]. Interestingly, there was a drop in the formation of follicular androstenedione, testosterone, and estradiol [9] where the enzymes catalyzing the formation of the latter hormones were located at the extra mitochondrial part of the cytoplasm. When immortalized granulosa cells were stimulated to

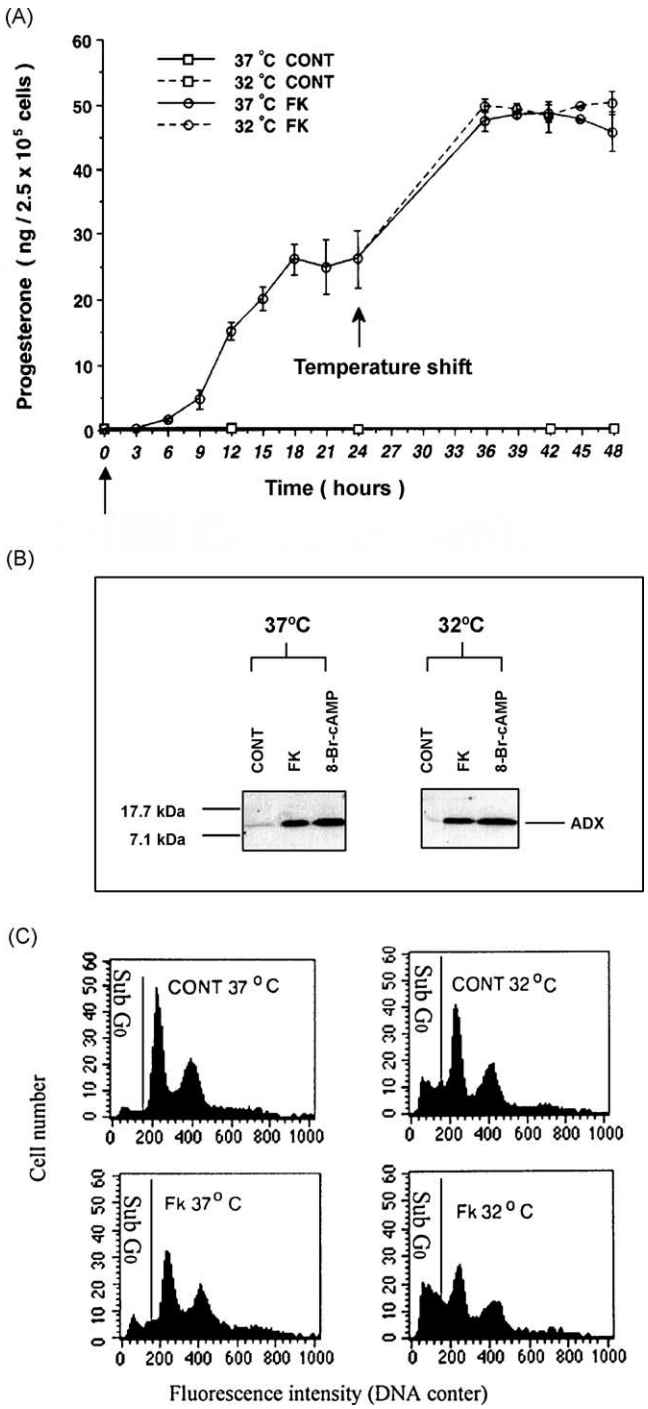

Fig. 2. Apoptosis and progesterone production in immortalized human granulosa cells. (A) Kinetics of progesterone production following stimulation with 50 μM forskolin (FK) from time 0. After 24 hr of culture a temperature shift from 37 to 32° stimulates apoptosis by activating the tumor suppressive and the apoptotic activity of p53, but in spite of stimulation of apoptosis, progesterone production and release are not attenuated in the cells containing a temperature sensitive mutant of p53, VAL 135 p53. (B) Intracellular levels of adrenodoxin (ADX) which is an integral part of the cytochrome p450scc does not decrease during induction of apoptosis. (C) Flow cytometry (FACS) analysis after temperature shift of ethidium bromide-labeled cells indicates massive increase of apoptosis as evident by the increase in the sub G0, cell fraction. Modified from [8,11] by permission.

undergo apoptosis by activation of p53 and stimulation with 50 μM of FK, there was a dramatic increase in progesterone production during 24 hr of incubation (Fig. 2). In order to verify whether the propagation of apoptosis and steroidogenesis can proceed in the same cell, the cells were double stained for DNA with DAPI or with the TUNEL method and with the Steroidogenic Acute Regulatory (StAR) protein, a typical mitochondrial marker for detecting steroidogenesis. We could clearly demonstrate the intactness of the mitochondria in cells undergoing apoptosis (condensed or fragmented nuclear DNA). This was verified also on the level of the electron microscope [8] (Figs. 4 and 5). It can thus be concluded that apoptosis and steroidogenesis can exist in the same cells until total cell collapse. Moreover, rearrangement of the actin cytoskeleton which leads to clustering of the intracellular organelles, which contain the steroidogenic enzymes can explain enhancement of progesterone production, such rearrangement and clustering allows for better coupling between the organelles involved in steroidogenesis (e.g. mitochondria and lipid droplets) [3,17].

4. Novel genes involved in regulation of apoptosis in granulosa cells

In order to verify which gene products may be involved in the protection of the mitochondria during initial stages of apoptosis, we performed a comprehensive screening of genes that in primary and immortalized granulosa cells are modulated by FSH, LH and FK, using hybridized mRNA extracted from treated and untreated cells on DNA microarrays of Affymetix, which covers a large portion of the rat and human genome [18,19]. We discovered that transrcriptosomes coding for granzyme-like proteins are elevated by gonadotropins and FK. We found similar phenomena using specific anti-Granzyme B antibodies in Western blot and immunocytochemistry. Moreover, we found that Granzyme B protein is dramatically accumulated and is cleaved to form active molecular species, which can directly activate a cascade of caspases that can bypass mitochondrial destruction [18–23]. Indeed, no cytochrome C release from granulosa cell mitochondria was evident during initial stages of apoptosis (Sasson and Amsterdam, unpublished). Also perforine that is responsible for the release of Granzyme B from granules was found to be expressed in the granulosa cells (Sasson and Amsterdam, unpublished). This is the first demonstration that granzyme-like proteins can reside and be released not via cytotoxic lymphocytes or natural killer cells.

Another protein, apoptotic repressor (ARC), which was uniquely found to reside in the heart muscle [24], was found to be expressed in granulosa cells.[1] This protein may significantly contribute to the protection of the follicular

[1] Sasson and Amsterdam, unpublished.

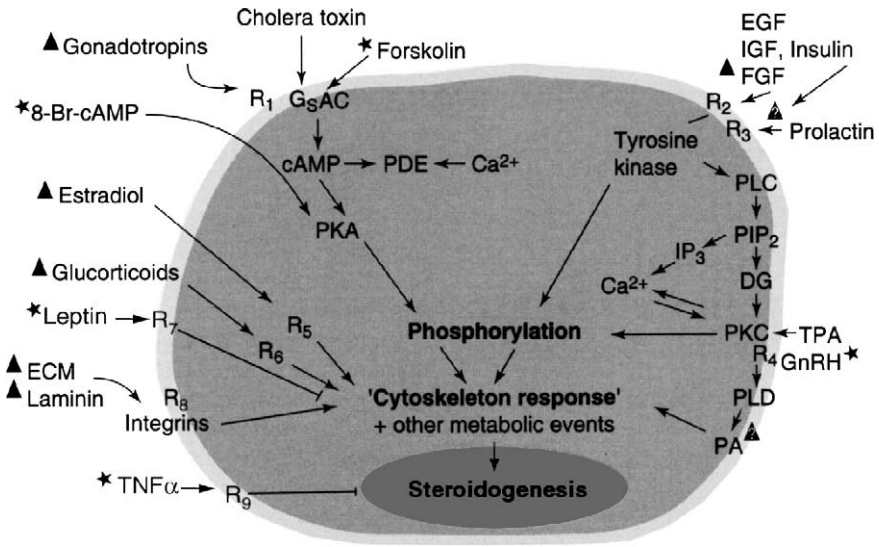

Fig. 3. Signal transduction pathways and possible crosstalk among signals that control steroidogenesis and programmed cell death in mature ovarian granulosa cells, and in immortalized steroidogenic granulosa cells. Stars indicate signals that may lead to both steroidogenesis and apoptosis; triangles indicate signals that may function as survival factors. The increased level of tyrosine phosphorylation [42] induced either by growth factors or by inhibiting tyrosine phosphatases (Pase) via vanadate was found to synergize with cAMP-generated signals in the induction of progesterone production [43]. Synergism in progesterone production between gonadotropin-releasing hormone (GnRH) stimulation, mediated at least in part by phospholipase D (PLD), and cAMP-generated signals was also reported recently [44]. Culturing of granulosa cells on an extracellular matrix, in the form of a native basement membrane, enhanced progesterone production by itself and augmented cAMP-induced steroidogenesis [7,36,45,46] in primary cells, while it attenuated cAMP-induced steroidogenesis in the immortalized cells. 'Cytoskeleton response' refers to the rearrangement of the actin cytoskeleton, which occurs during both luteinization [17,31] and apoptosis of granulosa cells [3,47] 'Other metabolic events' refers to upregulation of the steroidogenic enzymes, sterol carrier protein 2 (SCP2), the steroidogenic transcription factor SF1/Ad4-binding protein and the StAR protein (reviewed in [47]). DG, diacylglycerol; EGF, epidermal growth factor; IGF, insulin-like growth factor; IP3, inositol-1,4,5-trisphosphate; PA, phosphatidic acid; PDE, phosphodiesterase; PIP2, phosphatidylinositol-4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; R, receptor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; AC, adenylate cyclase, Gs, G-stimulatory protein (modified from [3]).

ovarian cells against mitochondrial destruction during apoptosis, since it contains a caspase recruitment domain and can preserve mitochondrial function.

5. Crosstalk among survival and death signals in granulosa cells

The possibility to culture purified primary granulosa cells and immortalized steroidogenic cells that express a temperature sensitive mutant of p53 (val135 p53) which behave as an oncogenic p53 at 37° and as an inducer of apoptosis at 32° allows for a comprehensive analysis of the molecules involved in crosstalk between signaling pathways for death and life in a well defined and synchronized apoptotic system [8,10,14]. Basic FGF exerts an anti-apoptotic activity, in general [25–28], and in particular a survival activity on p53-induced apoptosis, while cAMP augments p53-induced apoptosis [8]. We discovered that bFGF enhances MDM2 expression while cAMP attenuates MDM2 expression [3,8]. Therefore, since MDM2 is known to block p53 activity it can be concluded that MDM2 plays a pivotal role in the crosstalk between bFGF and p53 generated signals [8,29], and between cAMP generated signals and p53 signaling [3,8]. Another interesting crosstalk between the FGF receptor system and the p53–MDM2 system was recently revealed. When the FGFR3 G37R mutation for human dwarfness was introduced into mice by a knock-in technique, the mice became dwarfs and the females suffered from infertility. Analysis of the ovaries revealed absence of ovulation, and thus absence of corpora lutea formation and very intensive apoptosis in the granulosa cells that never reached stages of the Graafian follicle [30]. Interestingly, p53 intracellular levels in the dwarf mice were very high compared to normal animals while progesterone production was very low. These phenomena implicate malfunction of growth factor receptors with p53 expression, ovarian cell death and attenuation of progesterone production.

Glucocorticoids such as dexamethosone and hydrocortisone enhance steroidogenesis in granulosa cells while co-stimulation with gonadotropin/cAMP enhances production of progesterone [11,13,31]. Glucocorticoids were also found to exert a protective effect on apoptosis induced by serum deprivation, cAMP, p53 and TNFα [11,13,31,32]. We found that the protective effect is exerted by up regulation of Bcl2 and/or attenuation of its degradation [13,33]. Glucocorticoids, therefore, may play an important role *in vivo* by accelerating the healing process of the ruptured follicle subsequent to ovulation and during formation of the corpus luteum [34,35].

According to the information accumulated by us and other research groups, it seems that there are at least two pathways which regulate apoptosis in granulosa cells.

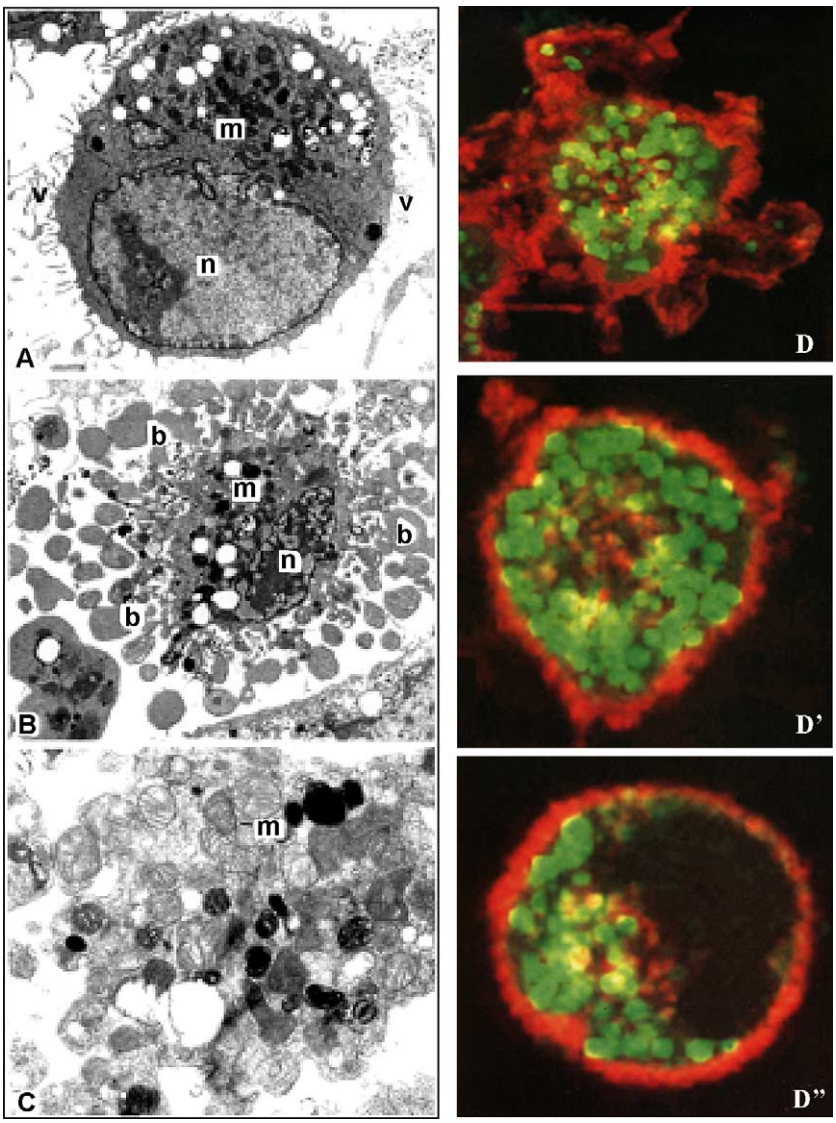

Fig. 4. Organization of intracellular organelles during apoptosis of immortalized steroidogenic granulosa cells. (A) Electron microscopic image of nonapoptotic immortalized human granulosa cell demonstrates numerous mitochondrial (m) and microvilli (v) on the cell surface. Magnification: 10,000×. (B) Apoptotic cell induced by activation of p53 demonstrating numerous cytoplasmic blebs (b). Chromatin in the nucleus that acquires irregular shape is condensed (n). Mitochondria remain intact in the central body of the cell. Magnification: 10,000×. (C) Enlargement of the central part of the cell. Mitochondria in the central part of the apoptotic cell contain well defined cristae and seem intact. Magnification: 30,000×. (D–D″) Apoptotic cell double stained with phalloidin rhodamine (red) for actin cytoskeleton and with antibodies to StAR mitochondrial protein (green). Three optical sections bottom (D″) middle (D′) and top (D) part of the cell. Note the rearrangement of actin cytoskeleton in the cell periphery (red) and the intactness of the steroidogenic mitochondria (green) which are highly clustered. Magnification: 3000×. Modified from Amsterdam *et al.* [3] and Hosokawa *et al.* [8].

The first one involves mitochondrial function, such as p53-induced apoptosis through Bax activation, or protection against apoptosis by leptin, glucocorticoid and TNFα through modulation of Bid and Bcl2. The other alternative apoptotic pathways are activated by cAMP through modulation of Granzyme B, or TNFα by activation of caspase 8 and downstream caspases 6, 3 or 7. Further investigation is needed in order to determine which of the alternative pathways is operated first. It seems likely that the alternative pathways which do not involve release of cytochrome C and mitochondrial destruction are operated first in order to maintain steroidogenic activity in the cells as long as possible. However, such a possibility must be investigated in more detail using short kinetics subsequent to apoptotic stimulation, combined with specific inhibitions to caspases and other components of the apoptotic apparatus.

6. The role of cell contact and intracellular communication in controlling apoptosis

Granulosa cells communicate both *in vivo* and *in vitro* via gap junctions, and they establish adherence junctions that are specialized zones of cell–cell contact [36–41]. It seems that the integrity of gap junctions plays an important

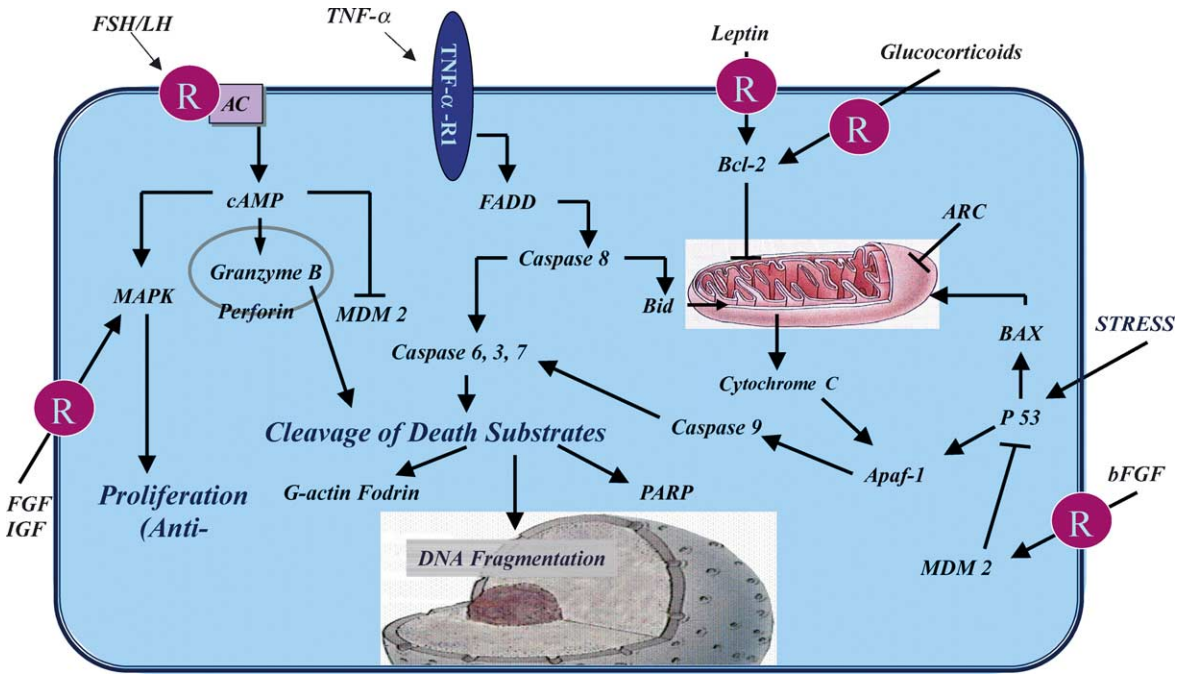

Fig. 5. Alternate pathways regulating granulosa cell apoptosis. Granulosa cell apoptosis can be stimulated by TNFα which can activate BID and the release of cytochrome C. It can also be activated by p53 which activates BAX and also leads to cytochrome C release. Glucocorticoid and leptin can protect mitochondrial destruction by up regulation of Bcl2, and bFGF can protect against p53-induced apoptosis by up regulation of MDM2. Mitochondrial destruction during these apoptotic signals can be protected at least in part by apoptotic repression (ARC) protein, which was recently discovered to be expressed in granulosa cells. In an alternative pathway, cAMP can induce apoptosis by activating Granzyme B, which can be released by perforine from secretory granules and directly cleave death substrates. Both gonadotropins, IGF and EGF can activate MAPK which negate the apoptotic signals.

role in the survival of granulosa cells. This conclusion is drawn from the fact that gap junctions become larger and appear in higher incidence subsequent to culturing of the cells on native ECM-like bovine corneal basement membrane [38] and/or in the presence of LH or FSH or in the presence of glucocorticoids [33]. Following stimulation for apoptosis, integrity of the junctions is interrupted [33], but it is not yet clear whether apoptotic signals cause the breakdown of gap junctions or whether breakdown of junctions initiates and accelerates the apoptotic process. Connexin 43 is a major component of the granulosa cell gap junction, and its expression is clearly elevated both by gonadotropins/cAMP [41] and glucocorticoids [33]. Adherence type junction size and frequency were also found to be elevated by glucocorticoids, concomitantly with the elevation of expression of cadherins (Sasson and Amsterdam, unpublished). Therefore, integrity of adherence and gap junctions may also play a role in the resistance of granulosa cells to apoptotic signals.

7. Conclusions

1. Ovarian cell death is critical for ovarian homeostasis.
2. Initial steps of this process enhance progesterone production by bypassing mitochondrial destruction.
3. Cytoskeleton rearrangement may be responsible for clustering of the steroidogenic organelles and for the temporal enhancement of steroidogenesis.
4. Novel components discovered to be expressed in granulosa cells, such as ARC and Granzyme B, may permit the protection of mitochondrial destruction during the apoptotic process, by bypassing mitochondrial apoptotic signaling, such as the release of cytochrome C.
5. Enhancement of progesterone release from apoptotic cells may sharpen the cyclicity of progesterone release in the estrus/menstrus cycle.

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