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REGULATION OF CELL DEATH AND CELL SURVIVAL GENE EXPRESSION DURING OVARIAN FOLLICULAR DEVELOPMENT AND ATRESIA

Jin-Yi Jiang, Carmen K.M. Cheung, Yifang Wang, Benjamin K. Tsang

Reproductive Biology Unit and Division of Reproductive Medicine, Department of Obstetrics & Gynecology and Cellular & Molecular Medicine, University of Ottawa; Hormones, Growth and Development Unit, Ottawa Health Research Institute, The Ottawa Hospital (Civic Campus) Ottawa, Ontario, Canada

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1. ABSTRACT

Mammalian ovarian follicular development and atresia is closely regulated by the cross talk of cell death and cell survival signals, which include endocrine hormones (gonadotropins) and intra-ovarian regulators (gonadal steroids, cytokines and growth factors). The fate of the follicle is dependent on a delicate balance in the expression and actions of factors promoting follicular cell proliferation, growth and differentiation and of those inducing programmed cell death (apoptosis). As an important endocrine hormone, FSH binds to its granulosa cell receptors and promotes ovarian follicle survival and growth not only by stimulating proliferation and estradiol secretion of these cells, but also inhibiting the apoptosis by up-regulating the expression of intracellular anti-apoptotic proteins, such as XIAP and FLIP. In addition, intra-ovarian regulators, such as TGF-alpha and TNF-alpha, also play an important role in the control of follicular development and atresia. In response to FSH, Estradiol-17 beta synthesized from the granulosa cells stimulates thecal expression of TGF-alpha, which in turn increases granulosa cell XIAP expression and proliferation. The death receptor and ligand, Fas and Fas ligand, are expressed in granulosa cells following gonadotropin withdrawal, culminating in caspase-mediated apoptosis and follicular atresia. In contrast, TNF-alpha has both survival and pro-apoptotic function in the follicle, depending on the receptor subtype activated, but has been shown to promote granulosa cell survival by increasing XIAP and FLIP expression via the IkappaB-NFkappaB pathway. The pro-apoptotic action of TNF-alpha is mediated through the activation of caspases, via its receptor- (i.e. Caspases-8 and -3) and mitochrondria- (i.e. Caspase-9 and -3) death pathways. In the present manuscript, we have reviewed the actions and interactions of gonadotropins and intra-ovarian regulators in the control of granulosa cell fate and ultimately follicular destiny. We have highlighted the role and regulation of granulosa cell XIAP and FLIP expression, as well as their interactions with the death signaling pathways in the maintenance of granulosa cell survival during follicular development. We have provided strong evidence for these intracellular survival factors as key determinants for ovarian follicular destiny (growth versus atresia), the expression of which is regulated by a highly integrated endocrine, paracrine and autocrine mechanism. Further studies in these aspects will lead to a better understanding of the molecular and cellular regulation of follicular development and atresia, and provide invaluable insight into novel strategies in assisted reproduction in human infertility as well as in increasing reproductive efficiency in livestock industries.

2. REGULATION OF FOLLICULAR DEVELOPMENT AND ATRESIA

Female mammals are endowed with a large number of primordial ovarian follicles at birth in spite of the large variability between species (from $2 \sim 4 \times 10^4$ in mice to $1 \sim 2 \times 10^6$ in human) (1). However, only a limited number of follicles develop to the preovulatory stage and ovulate, while most follicles (over 99%) undergo atresia during the course of folliculogenesis (2-4). Follicular

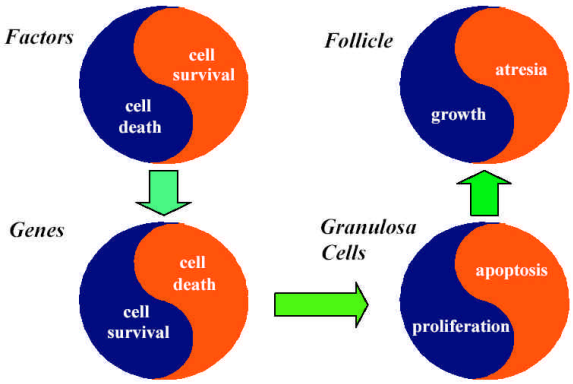

Figure 1. Ying-Yang hypothesis for the control of ovarian follicular growth and atresia.

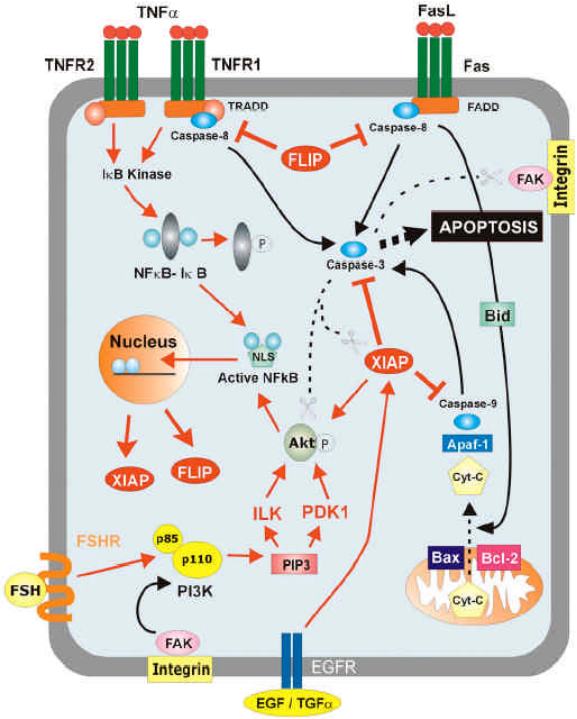

Figure 2. A hypothetical model illustrating the actions and interactions of FSH, TGF-alpha, TNF-alpha and Fas ligand, as well as integrin activation on cell death and cellular survival signaling in the granulosa cell.

development can be divided into three stages – recruitment of primary follicles, selection of the dominant follicles from the pool of antral follicles, and dominance (preovulatory follicles). It is believed that cohorts of primary oocytes are recruited to develop in early follicular phase. The initial stages of follicular development proceed very slowly and without follicular atresia (5). In contrast, follicular growth at the antral (penultimate) stage is much more rapid and follicles meet one of two fates (selection) – ovulation or atresia (6, 7). Although most follicles become atretic during the penultimate stage, a few are selected for dominance. In rats, primates and pigs, dominant follicles develop only during the follicular phase and are thus destined to ovulate. In cattle, sheep and horses, however, recruitment, selection and dominance occur at regular intervals, but only the dominant follicle (present during the follicular phase) ovulates (5).

The cellular and molecular mechanisms involved in follicular recruitment, selection and dominance are not well understood. Gonadotropins are known to play an important role in these processes and follicular recruitment is temporally correlated with slight increases in circulating FSH. Rats exhibit a secondary surge of FSH on the day of estrus, just before the next cohort of follicles is recruited (8). In primates, basal FSH is slightly higher at the beginning of the follicular phase than during the luteal or late follicular phases (9, 10). In some species, a decline in FSH levels for a defined period is necessary for follicle selection, since the dominant follicle (with increased abundance of FSH receptor) continues to grow in response to a low concentration of FSH, which is inadequate for survival and growth of the smaller follicles (11-13). FSH is also required for the maintenance of follicular dominance, since experimental reduction of plasma FSH during this period is correlated with cessation of growth in cattle, and in some animals the demise of the dominant follicle (5, 14).

3. REGULATION OF GRANULOSA CELL APOPTOSIS – A PHYSIOLOGIC MECHANISM FOR OVARIAN FOLLICULAR ATRESIA

The fate of the ovarian follicle (continual growth and ovulation versus atresia) at the penultimate stage of development is determined by the fate of its cells (proliferation and differentiation versus apoptosis), which in turn is regulated by the relative expression of “death” and “survival” genes under the influence of cell survival and cell death factors (Figure 1). Thus, follicular development may be a consequence of suppression of death genes and/or over-expression of survival genes, while the opposite is true for follicular atresia. Atresia is characterized by apoptosis initially of the granulosa cells (15) and subsequently of the theca (16). The onset of granulosa cell apoptotic cell death is evident morphologically by cytoplasmic and chromatin condensation, membrane blebbing and phagocytosis of neighboring cells (15, 17). Biochemically, this process is accompanied by the degradation of cellular DNA into nucleosomal units by Ca2+/Mg2+-dependent endonucleases (18, 19) and caspase-activated DNases (CAD) (20, 21). Follicular selection is closely regulated by endocrine, paracrine and autocrine factors (5, 22-24). FSH is an important survival factor for preantral and antral follicular development in vivo (25, 26). It promotes granulosa cell proliferation and estrogen secretion (24). Gonadotropin withdrawal by antibody neutralization (15, 27), hypophysectomy on day of proestrus or metabolic clearance after a single hormonal injection induces granulosa cell apoptosis and follicular atresia (28-30).

It is well established that apoptosis is triggered through the activation of cysteine aspartate-specific proteases (caspases; Figure 2), a family of the enzymes related to the interleukin-1 beta-converting enzyme (ICE,

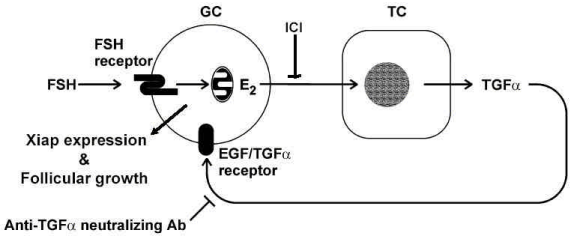

Figure 3. Granulosa-theca interaction in FSH-induced granulosa cell XIAP expression during follicular development (Reproduced with permission from Biology of Reproduction).

caspase-1) (31). During programmed cell death, caspases act as cell effectors and as initiators of apoptosis upon appropriate pro-apoptotic signaling. Caspases are expressed as inactive proenzymes and upon activation by autocatalytic cleavage or proteolytic signaling, they cleave a variety of substrates such as poly (ADP-ribose) polymerase (PARP), DNA-dependent kinase (DNA-PKs), as well as cytoskeletal cytoplasmic (actin, fodrin, laminin, beta-catenin and plakoglobin) and nuclear (lamin A, lamin B and Mdm2 oncoprotein) proteins (32-35). The rate and occurrence of substrate cleavage is cell-specific (36). Upon activation, caspase causes: a) inactivation of the inhibitors of apoptosis, b) disassembly of cellular structures, such as the nuclear lamina, c) deregulation of activity of key regulatory proteins, such as gelsolin (37), focal adhesion kinase (FAK) and p21-activated kinase 2 (PAK2) and d) cleavage of the inhibitor of CAD (ICAD), resulting in CAD release and translocation to the nucleus, and the cleavage of chromosomal DNA (20, 21, 38-40). In the ovary, caspase–3 expression appears to be regulated by gonadotropin and the activity of this protease is activated by upstream caspase-8 and –9 and act synergistically to cause granulosa cell death and hence, follicular atresia (41).

Three signaling pathways are involved in the induction of apoptosis: receptor- (Fas and TNFR), mitochondria- and endoplasmic reticulum (ER)-mediated death pathways, all of which involves the caspase activation. When bound to FasL, Fas initiates cell death by activating procaspase-8 and procaspase-3 (Figure 2). Mitochondria-mediated pathway is activated by many triggers, including growth factor deprivation. Cytochrome c released into cytoplasm forms a complex (apoptosome) with apoptotic protease activating factor-1 (Apaf-1), leading to the recruitment and activation of procaspase-9 and procaspase-3 (Figure 2) (42). In ER-mediated death pathway, ER stress triggers the activation of procaspase-12, which activates caspase-9 and subsequently caspase-3 in a cytochrome c-independent manner (43-45). Absence of or insufficient survival signaling within the granulosa cells (e.g. decreased FSH levels) will result in the entry of the follicle into an apoptotic suicide program, mediated by increased expression of death genes, such as Fas and Fas ligand (FasL) (46, 47). We have also demonstrated that FSH up-regulates XIAP level and suppresses apoptosis, thereby promoting growth of the dominant follicle (24). Taken together, the presence or absence of FSH appears to be critical in controlling the expression of pro-apoptotic and anti-apoptotic proteins in the ovarian follicles, an important determinant in follicular development.

In addition to gonadotropins, there are other factors that regulate cell survival and apoptosis in the ovary. Intra-ovarian regulators such as transforming growth factor alpha (TGF-alpha) (24) and tumor necrosis factor alpha (TNF-alpha) (48, 49) have been shown to increase the expression of intracellular cell survival intermediates, such as X-linked inhibitor of apoptosis (XIAP) and flice-like inhibitory protein (FLIP), and appear to enhance preovulatory follicular growth. Estradiol is a key regulator of TGF-alpha secretion from the theca cell and is instrumental in the up-regulation of follicular XIAP expression (24). Regulation of these proteins is multi-factorial and involves a delicate balance in the expression and action of cell death and cell survival factors to maintain a normal course of follicular development and atresia.

4. DEATH PATHWAYS IN THE INDUCTION OF OVARIAN APOPTOSIS

4.1 Fas and Fas ligand expression and the regulation of atresia

It is well established that the Fas/Fas ligand (FasL) system is involved in the induction of apoptosis in the ovary (Figure 2). Fas (APO-1/CD95), a 45-kDa transmembrane receptor, is a member of the TNF/nerve growth factor (NGF) receptor superfamily. When bound to FasL, the Fas receptor trimerizes and transduces a death signal, resulting in apoptosis (22, 50). FasL is a type II integral membrane protein structurally similar to TNF and lymphotoxin (50, 51). FasL is present in both membrane-associated and soluble forms and proteolytic cleavage of the membrane protein leads to the release of the soluble protein. Although the physiologic role of these two forms of FasL is unclear, they have been suggested to possess different receptor affinity and pro-apoptotic activity (52). In mammals, ligation of the Fas receptor results in activation of Fas-associated death domain protein (FADD), procaspase-8 and procaspase-3, and apoptosis (53). Fas transcripts have been isolated from thymus, liver, heart, lung, small intestine, kidney and testis (51, 54, 55). In the ovary, Fas and FasL are present in granulosa cells and are involved in the induction of apoptosis during ovarian follicular atresia (46, 56-61). Recently, we have co-localized Fas and FasL in atretic small and medium antral follicles in a pattern coincidental to the localization of TUNEL-positive cells and have demonstrated that gonadotropin withdrawal increased granulosa cell Fas and FasL content and induced apoptosis (29, 46). In addition, whereas granulosa cells isolated from immature rats exhibited high Fas and FasL content and extensive apoptosis, treatment of the animals with a single dose of eCG markedly decreased Fas and FasL levels and apoptotic cell death in 48-72 h (29). Cell cycle analysis showed significantly higher proportion of the gonadotropin-treated cells in S and G2/M phases compared to the untreated control. These latter responses appeared transient, as by 96 h post-eCG (when gonadotropin levels were markedly decreased by metabolic clearance) the expression of the death factors and the apoptotic response were again

evident. Coincidentally, the proportion of the cells was markedly increased in the A0 (apoptotic) and G0/G1 phases and decreased in S and G2/M, confirming an onset of apoptosis and the suppression of G1/S transition (29).

The p53 protein is an antiproliferative transcription factor that increases the rate of transcription of various genes involved in mitosis and apoptosis (62). It plays a critical role in cell cycle regulation (G1/S transition), DNA repair and induction of apoptosis (29, 63-65). In the ovary, changes in granulosa cell p53 content are correlated with those of Fas and FasL and is regulated by gonadotropins (22, 29). We have recently shown that p53 becomes markedly elevated during gonadotropin withdrawal, suggesting that induction of atresia is p53-dependent. Indeed, overexpression of p53 (by adenoviral p53 sense cDNA infection) resulted in increased Fas content and apoptosis, the latter response can be further augmented by the addition of an agonistic Fas antibody (29). Taken together, these findings demonstrate a central role of gonadotropin as a survival factor in the regulation of granulosa cell Fas, FasL and p53 expression during follicular development. They also suggest that the gonadotropic control of granulosa cell apoptosis involve two consecutive cellular events, cell cycle arrest at G1/S and exit from G0 into A0, via regulation of p53 and Fas/FasL death pathways. We have also demonstrated that the accumulation of the gatekeeper protein p53 at the cell cycle check-point (G1 phase) may play a significant role in the up-regulation of Fas necessary for G0 to A0 exit, granulosa cell apoptosis, and ultimately follicular atresia.

4.2. Mitochondrial and endoplasmic reticulum-associated death pathways

The mitochondria, through cytochrome c release, is involved in apoptotic signaling via one of the following mechanisms: a) interference with electron transport, oxidative phosphorylation and adenosine triphosphate production, b) release of caspase activator proteins, recruitment of Apaf-1 and subsequent procaspase-9 activation, and c) disruption of cellular reduction-oxidation reactions (66). The release of cytochrome c from the mitochondria can be inhibited by Bcl-2 (67, 68). Bcl-2 is a proto-oncogene that encodes a membrane-anchored intracellular protein that prevents apoptosis. The mechanism by which Bcl-2 blocks the release of pro-apoptotic proteins is based on its ability to regulate porous transport of cytochrome c (67). Other homologues of Bcl-2, such as oligomycin and buthionine sulfoximine, inhibit F0F1-adenosine triphosphatase proton pump activity and glutathione synthesis, respectively (66). BAX is a Bcl-2 homologous protein that modulates the action of Bcl-2 by blunting its heterodimer activity (69) or by acting independently (70-72) to induce apoptosis. Previous studies have demonstrated the presence of BAX mRNA and protein in the ovary and increased BAX expression in rat granulosa cells during apoptosis and at the onset of follicular atresia (73,74). These findings suggest that the Bcl-2 family members are important intracellular regulators of apoptosis and that a fine balance between Bcl-2 and BAX expression is maintained for granulosa cell survival during follicular development, which is modulated by cell death and survival signals.

The endoplasmic reticulum-mediated death pathway is a novel apoptotic pathway in which caspase-12 functions as the initiator caspase in response to a toxic insult (stress) to the endoplasmic reticulum, such as by treatment with thapsigargin (an inhibitor of the endoplasmic reticulum-specific calcium ATPase) or calcium ionophores (75). Furthermore, caspase-12-deficient cells are resistant to inducers of ER stress, suggesting that caspase-12 is significant in endoplasmic reticulum stress-induced apoptosis (44). Endoplasmic reticulum stress has received growing attention because of its implication in neurodegenerative disorders and the pathogenesis of diabetes (45,76). In the ovary, recent studies indicated that endoplasmic reticulum is associated with germ cell apoptosis and follicular atresia and is reorganized into perinuclear aggregates prior to perinatal germ cell loss (77). While dilation of smooth endoplasmic reticulum has been observed in the ooplasma in early atretic follicles (78), if and how this novel death pathway is involved in the regulation of apoptosis during follicular development and the induction of atresia, remains to be investigated.

Although the aforementioned cell death pathways can readily transduce a death signal to effect apoptosis independently, evidence indicates important cross-talk between them. In this context, Fas ligation is known to activate procaspase-8, which in turn causes the cleavage and activation of Bid, a pro-apoptotic member of the Bcl-2 family. The cleaved Bid induces cytochrome c release and activates the mitochondrial death pathway (42, 79, 80). Moreover, as these death pathways involve a coordinated series of procaspase activation, these enzymes also serve as important points of interaction with cell survival intermediates, so as to minimize or avoid premature or unwanted activation of apoptosis. Relevant to this aspect is their regulation by two families of intracellular survival proteins, often referred to as the inhibitor of apoptosis proteins (IAPs) and the FLICE-like inhibitory protein, which are reviewed in the following sections.

5. CELL SURVIVAL MEDIATED BY INHIBITOR OF APOPTOSIS PROTEINS

The inhibitor of apoptosis proteins (IAPs) constitute a family of highly conserved intracellular anti-apoptotic proteins. They were originally identified in baculovirus, where they maintained survival of the virus during replication (81, 82). Six members of the IAPs have been identified in mammals: neuronal apoptosis inhibitory protein (NAIP) (83), X-linked inhibitor of apoptosis protein (84), human inhibitor of apoptosis protein-1 (HIAP-1) (84), human inhibitor of apoptosis protein–2 (HIAP-2) (84), survivin and Livin (85, 86), which is also called KIAP in the kidney (87). These proteins share many structural similarities. The IAP family is characterized by the presence of the baculoviral inhibitor of apoptosis protein repeat (BIR), a ~70 amino acid motif, which confers biological activity to the IAP molecule (81, 82, 88). With the exception of NAIP and survivin, IAPs also have a C-terminal RING-Zinc finger domain, known to be important for protein-protein interaction (89, 90), and protein ubiquitination and degradation (90, 91). Functionally,

XIAP, NAIP and survivin has been shown to suppress apoptosis induced by various pro-apoptotic factors and conditions, including TNF-alpha, menadione, staurosporin, etoposide (VP16), Taxol, Fas activation and growth factor withdrawal (84, 85, 89, 92). In the ovary, IAP expression is a crucial element for follicular growth and survival and is controlled by gonadotropin. Previous studies in our laboratory have shown that extensive apoptosis of granulosa cells in preantral and early antral follicles is associated with reduced levels of HIAP-2 and XIAP (89). In contrast, administration of gonadotropin increases HIAP-2 and XIAP protein content and suppresses apoptosis in vivo. Conversely, withdrawal of gonadotropin support by administration of an anti-eCG antibody to eCG-primed prepubertal rats attenuates eCG-induced XIAP expression and increases DNA fragmentation, suggesting that IAPs play an important role in determining granulosa cell fate and thus, the destiny of the follicle (89).

The mechanism by which these anti-apoptotic proteins promote ovarian follicular cell survival remains an area of intense investigation and modulation of caspases is a well-accepted part of this physiologic process (Figure 2). Previous studies have shown that XIAP acts as a direct inhibitor of caspase-3 and caspase–7 (93, 94) and has the capability to suppress the mitochondrial (bax/cytochrome c-mediated) pathway via inhibition of caspase-9 (95). Additionally, as in the case of XIAP, HIAP-1 and HIAP-2 are thought to modulate TNF receptor signaling via binding to TNF Receptor Associated Factor-2 (TRAF2) and activating the Nuclear Factor kappa B (NFkappaB) pathway (96), thus resulting in the induction of inhibitor of apoptosis protein and subsequent suppression of caspase activities.

The inhibition of caspases by XIAP is modulated by three intracellular XIAP-interacting proteins: XIAP associated factor 1 (XAF1), Smac/DIABLO and HtfA2/Omi (97-101). XAF1 is a nuclear protein that negatively regulates XIAP by binding to it and inhibiting its anti-caspase activity. Smac/DIABLO and HtrA2/Omi are mitochondrial proteins, which are released into the cytosol and bind XIAP when the cell receives an apoptotic stimulus. They inhibit XIAP function and promoting a cytochrome c-mediated apoptotic response (99-101). The cytochrome-c complex recruits procaspase-9 and induces autoactivation (102, 103), leading to activation of various downstream procaspases (caspase-3, -6 and -7) and eventual apoptosis. The functional domains of Smac/DIABLO have been sequenced and are believed to reside in the first seven amino acids in its N-terminal (98, 100). HtrA2/Omi is a mitochondrial serine protease and, in addition to caspase activation, may function as a caspase-independent cell death inducer (99). Interestingly, nerve growth factor deprivation increases caspase-9 and DIABLO/Smac expression and induces neuronal apoptosis, suggesting that caspase-mediated pathways are dependent on the relative expression of its components (e.g. procaspases, IAPs, and IAP inhibitors), which are regulated by growth factors (104). Although the activity of these proteins has recently been established in cancer cell lines, their role and possible physiological regulation in the control of ovarian follicular growth and atresia is unknown.

6. REGULATION OF OVARIAN INHIBITOR OF APOPTOSIS PROTEIN EXPRESSION

6.1. Role of follicle stimulating hormone

Gonadotropins are important survival factors and have been shown to up-regulate IAP expression during follicle development in vivo (89). Moreover, gonadotropin withdrawal by antibody neutralization markedly decreases granulosa cell Xiap and HIAP-2 expression and induces apoptosis and follicular atresia (15, 89). When added to rat ovarian follicle cultures, FSH promotes granulosa cell proliferation, follicular growth and antrum formation (105, 106), a phenomenon also demonstrated in various species (5, 107-109). FSH also increases follicular XIAP expression in vitro. In the absence of the gonadotropin, follicles exhibit low XIAP expression, extensive apoptosis and minimal or no follicular growth in vitro, as judged by changes in DNA content, cell number and follicular volume. A definitive role of XIAP in the gonadotropic control of ovarian follicular survival and development has recently been confirmed in cultured follicles infected with adenoviral sense or anti-sense XIAP cDNA (24, 106). While minimal apoptosis is also evident in follicles cultured in the presence of low concentration of low FSH, significant follicular growth can be detected. Overexpression of XIAP by adenoviral sense cDNA infection completely suppresses apoptosis and promotes the gonadotropin-induced follicular growth. Conversely, infection of the cultured follicles with anti-sense XIAP can decrease FSH-induced XIAP contents, induce apoptosis and attenuate follicle growth induced by the gonadotropin (105). These results demonstrate that gonadotropin-induced XIAP expression plays a crucial role in the determining granulosa cell fate (survival or apoptosis), and thus follicular destiny (growth or atresia; Figure 2).

The signaling mechanism(s) involved in gonadotropin-induced XIAP expression during follicular development has only been recently investigated and the NFkappaB signaling pathway has been implicated (110). The NFkappaB family of transcription factors is involved in the up-regulation of XIAP gene expression in various cell types and is activated by various cytokines (49, 111-114). NFkappaB is composed of DNA-binding proteins (p65 and p50 subunits) essential for its activation. Activation of NFkappaB results in its translocation from the cytoplasm to the nucleus and in its binding to its responsive element in the promoter region of the gene for transcriptional activation. Recent studies from our laboratory have shown that addition of FSH to rat granulosa cell cultures results in rapid translocation of NFkappaB to the nucleus, increased NFkappaB-DNA binding activity and increased XIAP mRNA and protein contents. Pretreatment of the cells with SN50 [a cell permeable inhibitory peptide that binds and inactivates the nuclear localization signal (NLS)] but not its mutated inactive analogue SM50 (control peptide) prior to the gonadotropin challenge prevents NFkappaB translocation, suppresses NFkappaB activation and XIAP expression. When added to rat follicle cultures, SN50 also suppresses FSH-induced follicular growth, suggesting that NFkappaB activation is involved in the regulation of XIAP expression and follicular growth by the gonadotropin (110).

The cellular mechanism by which FSH activates NFkappaB and induces XIAP expression in the granulosa cell is not known. Our previous studies have shown that TNF-alpha increases XIAP expression in rat granulosa cells in vitro and that this response is mediated through activation of I kappaB kinase (IKK), resulting in I kappa B alpha (IkappaBalpha) phosphorylation and degradation, and the unmasking of the NLS of NFkappaB for its translocation to the nucleus (49). However, unlike TNF-alpha , FSH does not elicit IkappaBalpha phosphorylation and degradation in granulosa cells in vitro (110). Moreover, while over-expression of the dominant negative IkappaB (mutation of serine 32 and 36) can effectively attenuate TNF-alpha induced NFkappaB-DNA binding activity and XIAP expression, it is ineffective in suppressing these responses elicited by FSH, suggesting that FSH-induced NFkappaB activation is independent of IkappaB phosphorylation and degradation. We have recently shown that FSH increases granulosa cell phospho-Akt content and addition of the phosphotidylinositol 3-kinase (PI3K) inhibitors, LY294002 and wortmannin to granulosa cell cultures can markedly suppress FSH-induced NFkappaB activation and XIAP expression in a concentration-dependent manner (110). Taken together, these observations suggest that the FSH-induced, NFkappaB-dependent XIAP expression during follicular development is mediated through activation of the PI3K and not of the classical IKK pathway (Figure 2). The activation of the PI3K-Akt pathway can be directly or indirectly linked to NFkappaB activation (115, 116). The PI3K/Akt pathway does not contribute to the persistent induction of NFkappaB and transcriptional activation. In human endothelial cells, TNF-alpha and IL-1 activate a PI3K/Akt pathway and the anti-apoptotic effect of Akt is also independent of NFkappaB (117). These findings support the contention that NFkappaB-dependence of the PI3K cell survival pathway may be agonist- and cell type- specific.

The fate of the developing follicle is determined by the actions and interactions of endocrine and intra-ovarian regulators, which regulate granulosa cell fate via paracrine and autocrine mechanisms (25). In addition to gonadotropins, various factors synthesized and secreted within the follicles are known to have a direct action on granulosa cell proliferation, differentiation and apoptosis or to modulate gonadotropin control of these processes. It has been shown that the suppression of apoptosis by FSH is partially mediated through IGF-I (118) and that, in addition to FSH, epidermal growth factor (EGF) and IGF-I can stimulate follicle growth and antrum formation in vitro (119). EGF gene transcription is also involved in FSH-induced preantral development in the hamster ovary (120). Moreover, other intra-ovarian systems, including those of IGF and activin/inhibin, are also involved in follicle selection in response to the gonadotropin (121). Apoptosis in isolated granulosa cells cannot be abrogated by IGF-I, but IGF-I is effective in preventing cell death in whole isolated follicles (7). In the rat ovary, IGF-I is primarily synthesized in the granulosa cells (122, 123) and may bind to receptors on the theca to stimulate the release of EGF/TGF-alpha, which in turn promotes granulosa cell survival (124). However, our understanding of their possible involvement in the control of ovarian follicular XIAP expression by IGF-I during follicular maturation, remains incomplete.

6.2. Role of transforming growth factor alpha

Transforming growth factor alpha (TGF-alpha) is recognized as an important mitogenic (125-127), anti-differentiative (128), and antiapoptotic intra-ovarian factor in the regulation of granulosa cell function during follicular development (129). TGF-alpha, a member of the EGF superfamily, binds to the EGF receptor (EGFR) and promotes cell survival by activating the PI3K/Akt pathway in various cellular systems (130). In addition, it has been shown to stimulate granulosa cell proliferation during hen ovarian follicular development by increasing prostaglandin biosynthesis through the up-regulation of cyclooxygenase II and cytosolic phospholipase A2 (103, 127, 131). TGF-alpha is believed to be important in the programming of the transition of granulosa cells from a proliferative to a differentiated state and may determine the fate of developing follicles in the rat ovary (growth versus atresia) (128). In the human, TGF-alpha level in the follicular fluid is inversely correlated with follicle growth, suggesting that the importance of TGF-alpha as a mitogenic factor is follicular stage-dependent (132). TGF-alpha and EGF attenuate both the induction of aromatase by gonadotropin and follicular apoptosis (118, 133). TGF-alpha increases DNA synthesis in the undifferentiated but not differentiated granulosa cells in vitro (128). In addition, EGF and TGF-alpha prevent apoptosis in rat antral follicles and hen granulosa cells cultured in serum-free media through a tyrosine kinase-dependent mechanism (134, 135). Addition of EGF- or TGF-alpha-specific polyclonal antibody to ovarian follicle cultures can markedly attenuate FSH-induced ovarian follicular growth in both the hamster and the rat (24, 136, 137). These findings, together with our recent observations that FSH increases follicular growth in vitro but fails to stimulate proliferation of granulosa cells in primary cultures (24), support the notion that the mitogenic response of granulosa cells to FSH during follicular development may not be a consequence of the direct action of the gonadotropin, but rather of the synthesis and secretion of theca-derived factors, like TGF-alpha, which in turn suppresses apoptosis and promotes proliferation of the granulosa cells (24).

We have recently examined whether the FSH-induced XIAP expression and granulosa cell proliferation during follicular development is mediated by the secretion and action of intra-ovarian TGF-alpha (Figure 3). We have demonstrated that FSH stimulates estradiol production, TGF-alpha secretion, XIAP expression and follicular growth in rat follicles cultured for 2 and 4 days. In situ hybridization studies indicate that the theca cells are the primary follicular source of FSH-induced TGF-alpha (24). Intra-follicular injection of a neutralizing anti-TGF-alpha antibody or addition of estrogen antagonist ICI 182780 to the culture media suppresses FSH-induced XIAP expression and follicular growth. The effect of ICI 182780 is estrogen-specific and can be partially reversed by exogenous estradiol. TGF-alpha alone up-regulates XIAP content and granulosa cell proliferation in primary culture,

which can be effectively suppressed by the presence of anti-TGF-alpha antibody or by adenoviral XIAP antisense cDNA expression. In contrast, FSH alone is ineffective in eliciting the mitogenic response (24). Our results support the hypothesis that the FSH stimulates granulosa cell proliferation via theca TGF-alpha secretion and action in response to increased granulosa cell estradiol synthesis. XIAP up-regulation in response to FSH suppresses granulosa cell apoptosis and facilitates FSH-induced follicular growth. In addition, these studies demonstrate the participation of both cell types (i.e. granulosa and theca cells) in the FSH-induced, TGF-alpha-mediated granulosa cell survival and follicular growth (24).

6.3. Role of tumor necrosis factor alpha

As an important intraovarian regulator of steroid hormone production and follicular development and atresia (138-142), tumor necrosis factor alpha (TNF-alpha) is produced by several ovarian cell types including granulosa cells and the oocyte (143-146). TNF-alpha is a type II membrane protein (17.3-kDa) and is cleaved and solubilized by a membrane metalloproteinase (147). It is a multifunctional pleiotropic cytokine and is known to act via two distinct cell surface receptors, TNFR1 (55–60 kDa) and TNFR2 (75–80 kDa) (Figure 2) (148, 149). Although the extracellular domains of these receptors exhibit high sequence homology, their intracellular domains are distinct, indicating different signaling pathways are activated by ligand-receptor binding. Although TNFR1 is generally considered the predominant receptor and is mediated both apoptotic and survival signals (i.e. caspase-8/caspase-3 and NFkappaB, respectively) (150, 151), TNFR2 is primarily involved in survival signaling via NFkappaB (150).

The physiologic role of TNF-alpha in any cell will depend on a balance between apoptosis-inducing and cell survival signaling (152). It is thus conceivable that, in the ovary, the ability of TNF-alpha to promote follicular development or atresia will be dependent on the stage of follicular maturation, which in turn determines the relative abundance of its receptor subtypes and the expression of various intracellular death and survival factors (e.g. Fas/FasL, XIAP, FLIP, TGF-alpha) (48, 49, 58, 142). Although TNF-alpha has pro-apoptotic function and promotes Fas-mediated cell killing, it alone does not induce apoptosis in murine granulosa cells in vitro (49, 58). The binding of TNF-alpha to TNFRI leads to the cleavage and activation of procaspase-8 and procaspase-3 and of other downstream caspases, such as procaspase-7, an ultimate effector of apoptosis (153, 154). Further studies indicate that TNF-alpha induces apoptosis in granulosa cells from follicles at early developmental stages such as rat preantral and hen large white follicles (155, 156), suggesting TNFR1 is likely present in granulosa cells at these stages of follicular development. On the other hand, as a survival signal, TNF-alpha can induce intracellular survival factors for the maintenance of follicular development, especially in late follicle stages (48). TNF-alpha activates NFkappaB which mediates FLIP and XIAP expression, which promotes granulosa cell survivals by inhibiting procaspase-3, -7, -8 and -9 cleavage at the antral stage of follicular development (49, 142). We have recently demonstrated that the FLIP and Bcl-2 proteins are present in cultured rat granulosa cells and human ovarian surface epithelial cancer cells and are up-regulated by TNF-alpha in a NFkappaB-mediated mechanism (142).

6.4. Regulation of phosphatidylinositol 3-kinase/Akt pathway by XIAP

While the fate of the growing follicle is determined during the penultimate stage of development, the follicle maintains its viability until the time of ovulation once follicular dominance is established during the preovulatory stage. The cellular mechanism involved in the maintenance of follicle survival and thus of dominance, is not known. Activation of the PI3K/Akt pathway is important for the suppression of apoptosis in many cell systems, including granulosa cells during follicular development. Although XIAP is known to suppress granulosa cell apoptosis by inhibiting caspase activation and activities, if and how XIAP interacts with the PI3K/Akt pathway to promote granulosa cell survival and to maintain follicular dominance at this late stage of follicular maturation, has not been investigated. PI3K is composed of a catalytic (p110) and a regulatory (p85) subunit and is a lipid kinase which phosphorylates phosphoinoisitides (PtdIns) at position 3 of the inositol ring (157). PI3K is activated by diverse growth factor receptors and can phosphorylate Akt (also termed as Protein Kinase B or Rac kinase) via a serine/threonine kinase survival pathway (158), often referred to as the PI3K/Akt pathway (159-161). The activated Akt in turn phosphorylates and attenuates the actions of BAD, a pro-apoptotic member of the Bcl-2 family (162, 163). The phosphorylated BAD associates with 14-3-3 protein, preventing BAD-BCL-XL binding and promoting cell survival (164). Whether high levels of phospho-Akt and consequently of phosphorylated/ inactivated BAD is involved in preventing gonadotropin-primed granulosa cells from undergoing apoptosis during follicular development, is not known.

Using a fully characterized in vivo model to study the induction of follicular development and atresia in immature rats, we have demonstrated that gonadotropin treatment increases granulosa cell XIAP and phospho-Akt protein contents and suppresses apoptosis. In addition, gonadotropin withdrawal (eCG-primed rats treated with an anti-eCG antibody) can induce granulosa cell apoptosis and significantly decrease ovarian weight gain induced by eCG. The increased apoptosis is accompanied by marked decreases in XIAP expression and phosphorylation of Akt protein. While infection of granulosa cells from preovulatory follicles (eCG-primed rats) with adenoviral sense XIAP results in XIAP over-expression and increased phospho-Akt content, XIAP antisense expression decreases granulosa cell phospho-Akt level and induces apoptosis. Moreover, addition of the specific PI3K inhibitor LY294002 to the granulosa cell cultures decrease Akt phosphorylation and induces apoptosis in a dose-dependent manner. Taken together, these results demonstrate the importance and regulation of the PI3K survival pathway by XIAP in the control granulosa cell apoptosis and the maintenance of follicular survival (165), although the precise mechanism(s) involved remains to be determined.

Phosphoinosotide-dependent kinase (PDK-1) (166) and integrin-linked kinase (ILK, a serine and threonine protein kinase) can directly phosphorylate Akt and are therefore possible targets for XIAP action (167). Alternately, it is also possible that XIAP increases phospho-Akt content by down-regulating phosphatases (such as PTEN, a tumor suppressor protein and a phospholipid phosphatase) (168, 169). Since ILK is sensitive to and activated by high levels of PtdIns (3,4,5)P3 (167), mutation in the PTEN gene can lead to PtdIns(3,4,5)P3 accumulation and ILK activation. Another possible regulatory protein of PI3K is the Src homology 2 (SH2)-containing protein tyrosine phosphatase 1, which functions by direct association with its p85 subunit (170). However, which of these kinases and/or phosphatases are indeed putative candidates involved in the regulation of the phosphorylated (activated) Akt and survival in granulosa cells by XIAP, remains to be determined.

7. FLIP AS A CELL SURVIVAL FACTOR IN THE CONTROL OF OVARIAN APOPTOSIS

FLICE-like inhibitory protein (FLIP), an antiapoptotic factor, structurally resembles caspase-8 but lacks proteolytic activity (the cysteine residue within the active site) (171, 172). It exists in two different spliced isoforms, long ($FLIP_L$) and short (FLIPs), both of which contain two death-effector domains (DEDs) within their N-termini (171). The $FLIP_L$ isoform includes an additional C-terminal structure resembling the p20 and p10 subunits of caspases (173). FLIP is recruited to the death-inducing signaling complex (DISC) through the adaptor molecule, Fas-associated death domain (FADD), thereby preventing the recruitment of procaspase-8 into the complex and its subsequent activation, which then suppresses apoptosis (Figure 2; 142, 174). Information regarding the expression, regulation and role of FLIP in the ovary is limited. It has been demonstrated that the transgenic c-FLIP-/- mouse embryo rarely survives past Day 11 of embryogenesis and that their fibroblasts are highly sensitive to FasL- or TNF-alpha-induced apoptosis (175).

We have recently cloned and sequenced a full-length rat ovarian FLIPs gene, which share high homology to the mouse counterpart (85%), and have examined its role and regulation by TNF-alpha in the rat granulosa cells (142). While TNF-alpha has no apparent effect on steady state levels of $FLIP_L$ mRNA in rat granulosa cells in vitro, FLIPs mRNA abundance rapidly increases in the presence of the cytokine. A significant elevation in FLIPs mRNA levels is evident within 1 h of TNF-alpha treatment and is sustained for at least 6 h. Treatment of granulosa cells with TNF-alpha results in a rapid but transient increase in phospho-IkappaB levels and NFkappaB activation. SN50 (an inhibitor of NFkappaB translocation), but not its inactive peptide SM50, can markedly attenuate TNF-alpha-induced NFkappaB activation, as evident by suppressed nuclear NFkappaB binding activity. These responses are coincidental to a marked decrease in TNF-alpha-induced FLIPs mRNA expression, whereas $FLIP_L$ mRNA expression is not affected by any of the treatment. In addition, expression of FLIPs antisense cDNA in granulosa cells blocks the TNF-alpha-induced increase in FLIPs protein content. Down-regulation of FLIPs expression can significantly increase TNF-alpha-induced apoptosis. These findings suggest that, in addition to its pro-apoptotic function, TNF-alpha can induce an intracellular survival factor for the maintenance of follicular development. TNF-alpha-induced, NFkappaB-mediated FLIPs expression is a determinant of granulosa cell fate (142).

8. FUTURE RESEARCH DIRECTIONS

The advent of state-of-the-art molecular and cellular technologies in recent years and their applications to reproductive sciences as well as the availability of recombinant bioactive peptide preparations have advanced our understanding of the role and regulation of cell death and cell survival factors in the control of follicular development and atresia. While we have just begun to unravel the mystery of individual cell death and survival signaling pathways in these processes, one cannot but be amazed by the complex interactions between these control mechanisms and how the cross-talk of the different signaling pathways ultimately determines the destiny of the developing follicles. While these new knowledge have led to unparalleled advancements in human and veterinary reproductive medicine, as evident in the past decade, by marked improvement on reproductive efficiency in animal production programs and new therapeutic strategies in assisted reproduction in the human, considerable gaps remain in our current understanding of the precise mechanism(s) by which the fate of ovarian cells is regulated during follicular development and reproductive aging.

Recent evidence clearly demonstrates that activation of specific TNFR subtypes is associated with signaling of both death and survival pathways, the consequence of which is the activation of caspases and NFkappaB (with increased XIAP and FLIP expression), respectively. It is conceivable that the predominance of either of these pathways will in part be influenced by the relative abundance of the TNFR subtypes. However, the relative expression of TNFR subtypes during follicular development, their signaling mechanism (s) involved as well as their control by gonadotropins and intra-ovarian regulators, is as yet unknown. Further investigations on these aspects will provide important information if and how TNF-alpha and TNFR subtypes participate in follicle selection and dominance during follicular development.

In addition, while this review has clearly shown that the steady-state level of XIAP in granulosa cells is dependent on the expression of the XIAP gene in response to endocrine (e.g. FSH) and intra-ovarian (e.g. TNF-alpha and TGF-alpha) regulation, increasing evidence in other cellular system suggest that the processing/degradation of XIAP may also be important. It has been demonstrated that XIAP, while being an endogenous inhibitor of caspase-3, is also an endogenous substrate of this death protease (176-178). However, whether this phenomenon is physiological relevant to the regulation of ovarian follicular survival and dominance, has not been examined. These observations

raise the interesting possibility that the fate of the granulosa cells, and thus follicular destiny, may be determined by the relative intracellular levels of these interactive intracellular proteins, which is tightly controlled by the microenvironment of the follicles. Studies on how FSH and intra-ovarian factors interact in maintaining the delicate balance between XIAP level and caspase content as well as activation will provide important information on regulatory mechanism in follicular development and atresia.

Recent evidences suggest that XIAP content may also be regulated by post-translational modification and degradation. It has recently been reported that the RING zinc finger domain of XIAP possesses an ubiquitin protein ligase (E3) (179-181), which may be important in controlling the levels of other intracellular proteins and in XIAP self-ubiquitination and auto-degradation in thymocytes in response to apoptotic stimuli (91, 182). Several E3s are highly homologous to IAP with respect to their RING finger domains and promote degradation of both themselves and specific substrates such as p53 (179). Recent studies also indicate that XIAP promotes ubiquitination of caspase-3 and –7 (178, 183), raising another possible level of control of caspase function by XIAP. The possibility of XIAP auto-ubiquitination as an important regulatory mechanism in the granulosa cell growth and apoptosis has not been investigated. Moreover, whether caspase ubiquitination and degradation indeed serves as a physiologic means by which XIAP modulates caspase function in granulosa cells and how these processes are control during follicular development, remain to be examined.

The expression of XIAP in granulosa cells is up-regulated by FSH and TNF-alpha (49, 105). However, recent evidence suggests that the function of XIAP is negatively modulated by a group of intracellular caspase activators, such as DIABLO/Smac, XAF1 and HtfA2/Omi. These proteins suppress the caspase inhibitory activity of XIAP through binding to either or both of the bacculovirus IAP repeats (BIR2 and BIR3) domain of XIAP (184, 185). Our recent studies have demonstrated that while the high expression of XIAP in granulosa cells was associated with suppressed apoptosis in late stages of follicular development, no clear relationship appear to exist between follicular XIAP content and apoptosis at the preantral stage (89). Whether these XIAP regulators are expressed in a follicular stage-dependent manner and play a significant role in follicle selection (early stages of follicular development) or in the maintenance of follicular dominance of the selected follicle(s) (during the antral stage), is not known. Investigation into this aspect will provide a better understanding of their role in regulation of granulosa cell fate and ultimately follicular destiny.

Increasing evidence indicates that environmental toxins have adverse effects on human reproduction. Recent studies have shown that exposure to environmental toxicants, such as dioxin and polychlorinated biphenyls, are associated with decreased male fertility and increased pregnancy failure (186-192). Our understanding of the mode of action and the adverse outcome of these compounds in the male has significantly been improved, due in part to improved sensitivity of the analytical techniques for these toxins and the tightly synchronized nature of the developmental process of the male germ cells, thus allowing the definition of the stage(s) of spermatogenesis that is most sensitive to the toxic insults. In contrast, while in vivo models have been used frequently to examine their potential adverse effects on ovarian function, the presence of pools of follicles at different stages of follicular development in the ovary and potentially with different sensitivity renders the interpretation of these findings difficult. As a consequence, the biochemical and cellular mechanism(s) by which environmental toxins influence female reproductive health as well as their target cells remains poorly understood. The recent development of an in vitro follicle culture system offers a new and convenient approach by which potential adverse effects of toxicants of interest at physiologically relevant concentrations on follicular development and function may be studied in much greater details than previously possible (193). In this context, the ability to monitor morphologically, biochemically and functionally their influence on the oocytes and the support cells (granulosa cells and theca cells) of individual or a pool of follicles of clearly defined stages of development will facilitate the assessment of follicular stage- and cell type-specific action of these toxicants. In addition, the in-vitro rat follicle culture system recently established in our laboratory has been proven to be an excellent model to investigate the regulatory mechanisms involved in granulosa cells apoptosis and cell survival during follicle development and atresia (24, 105, 110). This in vitro system will also be helpful for the assessment of not only on the effect of these toxins on the fate of these support cells, the signaling pathways involved, but also of their influence on the quality of the oocytes, be it directly or indirectly. These studies will provide important insight on whether environmental toxicants of interest adversely affect ovarian function, including infertility, premature ovarian failure and ovarian cancer.

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Send correspondence to: Benjamin K. Tsang, Ph.D., Ottawa Health Research Institute, The Ottawa Hospital (Civic Campus), 725 Parkdale Avenue, Ottawa, Ontario, Canada K1Y 4E9, Tel: 613-798-5555 Ext. 16040, Fax: 613-761-4403, E-mail: btsang@ohri.ca