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# Apoptosis during folliculogenesis in pigs

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The number of female germ cells in pig fetuses decreases by 70% between day 50 after mating and day 300 after birth. Approximately 55% of antral follicles undergo degeneration (atresia) except during the 3 days before oestrus, when only 15% of the follicles survive to ovulate. Apoptosis, a form of programmed cell death, is recognized as the mechanism of germ cell death and follicle atresia at all stages of folliculogenesis. The internucleosomal cleavage of genomic DNA caused by caspase-induced deoxyribonuclease activity was measured in pig granulosa cells by DNA fluorescence flow cytometry, densitometry of fluorescently labelled internucleosomal DNA fragments and immunohistochemical analysis of the 3′ end labelling of deoxyribonuclease-nicked DNA on frozen tissue sections. Follicular atresia during the 3 days before oestrus is associated with a 60–70% decrease in the secretion of FSH. In granulosa cells, apoptosis is associated with decreased cell proliferation and reduced production of oestradiol and inhibin. In cultured pig granulosa cells, FSH and IGF-I are anti-apoptotic and a caspase inhibitor blocked apoptosis, thereby providing evidence of caspase activity. Oocytes in most follicles have resumed meiotic maturation; therefore, one role for apoptosis and follicle atresia may be to act as a barrier to ovulation of oocytes that have not remained in meiotic arrest.

## Mechanism of apoptosis

Apoptosis or programmed cell death is a physiological process that is essential for the successful development and survival of multicellular organisms (Vaux, 1993). The distinguishing characteristic of apoptosis is the ordered disassembly of cells from within a remodelling process required to remove cells that have completed their functions and are no longer needed, or that fail to develop properly, or that are damaged genetically. Apoptosis is triggered by environmental or physiological stimuli, or by withdrawal of trophic factors. As the predominant form of physiological cell death in eukaryotic cells, apoptosis requires energy utilization and is often regulated at the level of transcription or translation. These features of apoptosis distinguish it from necrosis, a pathological form of cell death, which is a result of injury and is characterized by swelling of the cell, activation of an immune response and lysis (Arends *et al.*, 1990).

Physiological induction of apoptosis follows two general pathways: negative induction by survival factor withdrawal (Fig. 1a) and positive induction by specific ligand binding to a plasma membrane receptor (Fig. 1b). Six major components in these pathways have been

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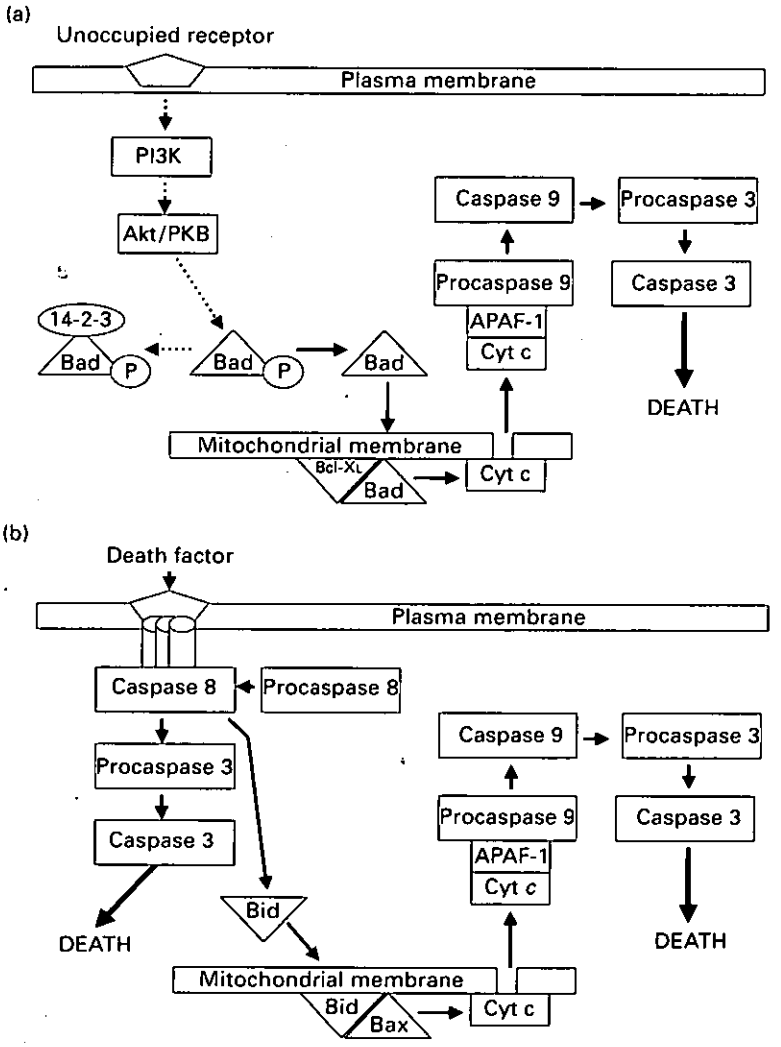


**Fig. 1.** Model for apoptosis pathway in pigs. (a) Negative induction by withdrawal of trophic or cell survival factors and (b) positive induction by specific ligand binding to plasma membrane receptors. In the negative induction pathway, receptor occupancy maintains a phosphorylation signal transduction pathway to sequester phosphorylated pro-apoptotic B-cell lymphoma/leukaemia-2 (Bcl-2) family proteins, such as Bcl-$X_L$/Bcl-2-associated death promoter (Bad) in the cytosol. If the receptor for a survival factor, such as FSH or insulin-like growth factor (IGF-I), is vacated then the phosphorylation signal transduction pathway is disabled; Bad is dephosphorylated and translocated to the mitochondrion. As a consequence of binding with an anti-apoptotic protein, such as Bcl-2 and Bcl-2-related gene-x long form (Bcl-$X_L$), the outer mitochondrial membrane is destabilized and cytochrome *c* (Cyt *c*) escapes to initiate caspase activation. In the positive induction pathway, ligands, such as tumour necrosis factor, Fas and angiotensin II, occupy their membrane receptors to cause aggregation (trimerization) of plasma membrane receptors and orient receptor cytosolic-death domains into a configuration that recruits adaptor proteins to promote the binding and activation of procaspases. Pro-apoptotic Bcl-2 proteins localized in the

described to execute pro-apoptotic or anti-apoptotic processes in mammalian species. These components are: (i) plasma membrane receptors for cell survival factors and cell death factors; (ii) the B-cell lymphoma/leukaemia 2 (Bcl-2) family of proteins; (iii) cytochrome *c*; (iv) apoptotic protease-activating factor 1 (APAF-1); (v) cysteinyl aspartate-specific proteases (caspases, interleukin-1β-converting enzyme family); and (vi) inhibitory apoptosis proteins (IAP) (for reviews, see Ashkenazi and Dixit, 1998; Thornberry and Lazebnik, 1998; Desagher and Martinou, 2000; Guthrie and Garrett, 2000a).

Results from other studies indicate that the mitochondrion is a major target for the Bcl-2 proteins (Desagher and Martinou, 2000). Currently, 24 Bcl-2 proteins have been identified in mammals. Each member of the family contains at least one of four regions of Bcl-2 homology that permit Bcl-2 proteins to form homo- and heterodimers to regulate each other. The Bcl-2 proteins possess either anti-apoptotic or pro-apoptotic function. For the most part, the anti-apoptotic members bind to the outer mitochondrial membranes and the pro-apoptotic members are distributed in both the cytosol and mitochondria. The primary mechanism by which Bcl-2 proteins regulate apoptosis is by controlling the release of cytochrome *c* from the mitochondrion into the cytosol, where it coordinates the activation of caspases. The release of cytochrome *c* from the mitochondrion initiates the 'caspase cascade' and serves as the critical point of control for apoptosis. In the cytosol, cytochrome *c* forms a complex with APAF-1, dATP and procaspase 9 resulting in proteolytic activation of 'initiator' caspases, such as caspase 9. Caspase 9 subsequently activates downstream 'effector' caspases, such as caspase 3, which functions to degrade cellular components or activate other substrates (Fig. 2) by proteolytic attack (Enari *et al.*, 1998).

Caspases are responsible for the inactivation or activation of protein activities that lead to the death or disassembly of affected cells, and are present as inactive pro-enzymes, most of which are activated by proteolytic cleavage themselves (Cohen, 1997; Miller, 1997). The execution phase of caspase activity is represented by caspase 3 which, serving as a substrate for initiator caspases, amplifies the earlier cell death signals, resulting in cleavage of vital cellular proteins for completion of the cell death pathway (Fig. 2).

The specific substrate targets of the execution phase of apoptosis that affect the final disassembly of the cell are varied. The actions and substrates include disruption of microfilaments (GAS2), cytoskeletal components (actin and fodrin), chromosome structure (lamin), the cell cycle (retinoblastoma protein), DNA repair (poly[ADP]-ribose polymerase), low density lipoprotein receptor expression (sterol regulatory element-binding proteins 1 and 2) and signal transduction (catalytic subunit of DNA protein kinase). The chromatin in the nucleus is supported by a scaffold of protein designated as the nuclear matrix. The final stages of apoptosis are characterized by the activation in the nucleus of a deoxyribonuclease, which cleaves DNA between nucleosomes into internucleosomal fragments.

The IAP family are cytoplasmic proteins that bind to, and inhibit the activity of, specific caspases that function in the cell death pathway (Roy *et al.*, 1997).

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cytosol, such as Bcl-2-related death gene (Bid), are activated by caspase action mediated through the Fas/tumour necrosis factor apoptosis signal and are translocated to the mitochondrion for heterodimerization with other proapoptotic proteins such as Bcl-2 associated-x-gene (Bax) to destabilize the outer mitochondrial membrane resulting in the release of cytochrome *c*. The positive induction pathway can initiate a 'caspase cascade' that results in a death process that can be independent of or dependent on changes in mitochondrial stability. Akt: serine-threonine kinase; APAF-1: apoptotic protease-activating factor 1; PI3K: phosphatidylinositide-3-OH kinase; PKB: protein kinase B.

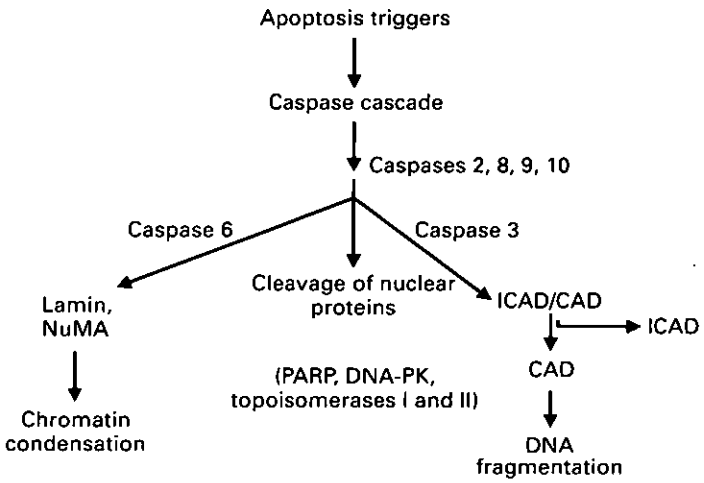


**Fig. 2.** Model for the role of caspases in nuclear apoptotic events showing the specific substrate targets of the execution phase of apoptosis leading to cleavage of genomic DNA into internucleosomal fragments. CAD: caspase-activated DNase; DNA-PK: DNA-dependent protein kinase; ICAD: inhibitor of CAD; NuMA: nuclear-mitotic apparatus protein; PARP: poly(ADP-ribose) polymerase.

## Folliculogenesis and apoptosis

In pigs, the number of germ cells per female reaches a maximum of $1.2 \times 10^6$ in the fetus at day 50 after mating and then decreases sharply to $5.0 \times 10^5$ at parturition (Fig. 3). Germ cell development is dynamic in the fetus with proliferation, meiotic arrest, primordial follicle formation, primordial follicle activation and germ cell death occurring simultaneously in the same ovaries. In the ovaries of pig fetuses, about 5% of oocytes are undergoing apoptosis as indicated by the presence of pyknotic nuclei (Black and Erickson, 1968). Although germ cell death has not been studied extensively in pigs, in laboratory species it has been established that during early stages of oogenesis and folliculogenesis, apoptosis plays the primary role in oocyte degeneration (Pesce *et al.*, 1993; Pesce and De Felici, 1994). Bcl-2 knockout mice have reduced numbers of primordial follicles (Ratts *et al.*, 1995), and targeted overexpression of Bcl-2 in mouse ovarian somatic cells leads to reduced follicular atresia and increased litter size (Hsu *et al.*, 1996). After parturition, the number of germ cells per female decreased at a slower rate to $2.0 \times 10^5$ at day 300 of age (Fig. 3). In pigs, preantral follicle growth begins within 10 days of birth (Erickson, 1967). Antral follicles are first observed histologically at about day 65 of age and emerge from the ovarian surface at about day 80 of age (Erickson, 1967; H. D. Guthrie, unpublished). We found that Bcl-2 was not expressed in germ cells and that expression was greatest in stromal cells located in the ovarian cortex surrounding primordial follicles and in the granulosa cells of primordial and preantral follicles (Garrett and Guthrie, 1999). This finding indicates that Bcl-2 mediates its survival effect indirectly through stromal cell survival factors.

Granulosa cell apoptosis is the underlying molecular mechanism of follicular atresia (Hughes and Gorospe, 1991; Tilly *et al.*, 1991; Guthrie *et al.*, 1995). The incidence of follicular atresia, determined by pyknotic nuclei in granulosa cells, is lowest among primordial follicles ($\leq$ 6%) and preantral follicles ($\leq$ 17%), and greatest among antral

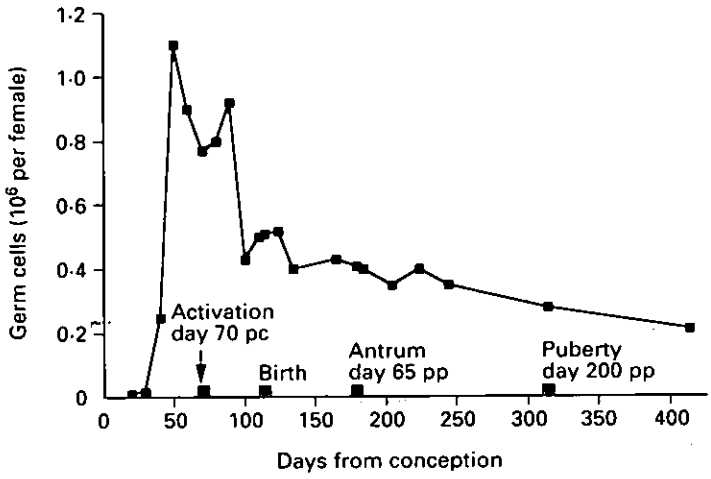


**Fig. 3.** Changes in the number of germ cells during folliculogenesis in female pigs showing key stages in development. Primordial follicle activation was first detected at day 70 post coitum (pc) and follicle antrum formation was first detected at day 65 post partum (pp) (modified from Erickson, 1967; Black and Erickson, 1968).

follicles, averaging 55% in pre- and postpubertal gilts (Erickson, 1967; Dalin, 1987). The formation of antral follicles and the increased incidence of atresia during prepubertal development is coincident with a 60% decrease in mean plasma concentrations of FSH and LH between day 60 and day 120 of age (Guthrie and Garrett, 2000a).

## Ovarian regulation of atresia and apoptosis

Biochemical characteristics of atresia and apoptosis have been investigated to discover causative factors and markers in an attempt to identify follicles in early stages of atresia. Results from studies of ovarian gene expression *in vivo* and *in vitro* have indicated that ovarian products, such as steroid hormones, transforming growth factor family members, insulin-like growth factor I (IGF-I), IGF-binding proteins and inhibins could play a role in the atretic process and apoptosis.

### *Steroid hormones*

Follicles that contain ⩾ 10% of apoptotic granulosa cells were found to have very low follicular fluid concentrations of oestradiol (1–5 ng $ml^{-1}$; Guthrie *et al.*, 1995). Among non-atretic follicles, oestradiol concentrations were inversely related to the percentage of apoptotic (%$A_0$) granulosa cells ($r = -0.45$; $P = 0.01$; Fig. 4) at the beginning of a follicular phase, on day 1 after withdrawal of altrenogest and on day 6 of the oestrous cycle (Guthrie *et al.*, 1995). It was concluded that low follicular fluid concentrations of oestradiol are a very early predictor of atresia. Loss of aromatase activity and enzyme protein (Guthrie and Garrett, 2000a) in granulosa cells is an important characteristic of atresia in follicles and may be mediated, in part, at transcription (Tilly *et al.*, 1992a).

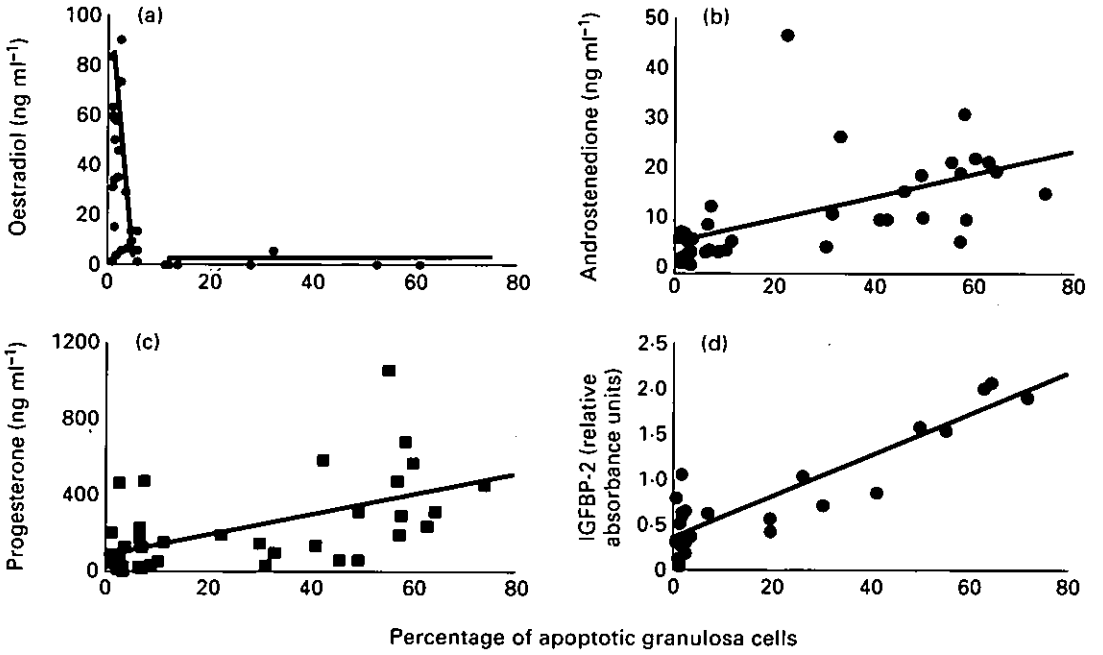


**Fig. 4.** Linear regression of follicular fluid concentrations of (a) oestradiol, (b) androstenedione, (c) progesterone and (d) IGF binding protein 2 (IGFBP-2) on granulosa cell apoptosis in individual medium-sized nonatretic (< 10% apoptotic granulosa cells) and atretic (≥ 10% apoptotic granulosa cells) follicles in pigs (modified from Guthrie *et al.*, 1995).

Progesterone and androstenedione content of atretic follicles is similar to, or greater than, that of nonatretic follicles on days 6–7 of the oestrous cycle (Guthrie *et al.*, 1995). Androstenedione and progesterone is were produced continuously *in vivo* in the follicular fluid as follicles became increasingly atretic as indicated by a greater proportion of apoptotic granulosa cells (Fig. 4). These results are in agreement with findings indicating that atretic pig follicles in culture release similar or greater amounts of androgen or progesterone to healthy follicles (Guthrie *et al.*, 1995). The ability of atretic follicles to continue production of androgens and progesterone is probably due to the ability of theca cells to retain specific binding and steroid responsiveness to LH even in advanced stages of atresia, whereas specific binding of FSH in granulosa cells disappears relatively early during atresia (Tsafriri and Braw, 1984). The loss of specific binding of FSH in granulosa cells of atretic follicles may account, in part, for the loss of aromatase activity. Similarly, granulosa cells from atretic pig follicles were no longer able to release cAMP in response to exogenous gonadotrophins (Guthrie and Garrett, 2000a). In small pig follicles, expression of LH receptor and FSH receptor transcripts was localized to the theca interna and granulosa cells, respectively (Guthrie and Garrett, 2000a). In atretic follicles, LH receptor was expressed weakly in theca cells, whereas there was no expression of FSH receptor in granulosa cells (Guthrie and Garrett, 2000a).

Steroids modulate apoptosis in cultured granulosa cells of rats (Hsueh *et al.*, 1994). Progesterone acting through a novel membrane receptor was shown to inhibit apoptosis in cultured granulosa cells of rats (Peluso and Pappalardo, 1994). In addition, the anti-apoptotic effect of epidermal growth factor (EGF) might also act by stimulating progesterone production. Progesterone production by pig granulosa cells *in vitro* was inversely related to apoptosis in the presence of FSH or IGF-I (Guthrie *et al.*, 1998). This is in contrast to the positive correlation

between follicular fluid concentrations of progesterone and the percentage of apoptotic granulosa cells during early stages of atresia *in vivo* (Guthrie *et al.*, 1995).

In an attempt to clarify the role of progesterone in relation to apoptosis, the relationship between apoptosis and progesterone production in pig granulosa cells was examined using inhibitors of progesterone production and apoptosis. Although trilostane inhibited FSH-induced progesterone production, it did not affect the anti-apoptotic effect of FSH. When apoptosis was inhibited using the general caspase inhibitor benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethylketone (Guthrie *et al.*, 2000), no effect on progesterone production was found. These results indicate that apoptosis and progesterone production are regulated independently by FSH in pig granulosa cells. In addition, although EGF also increased progesterone production in cultured pig granulosa cells, it did not have an anti-apoptotic effect (H. D. Guthrie, unpublished).

Atresia is regulated by oestrogens and androgens in laboratory species (Tsafriri and Braw, 1984). Oestradiol benzoate was shown to block apoptosis in granulosa cells after the withdrawal of diethylstilbestrol (DES) in hypophysectomized, DES-treated, immature female rats (Billig *et al.*, 1993). Furthermore, simultaneous treatment with testosterone prevented the anti-atretogenic effect of oestradiol benzoate, indicating that an interaction between locally produced oestrogen and androgen could regulate processes that trigger apoptosis and atresia. Further studies are required to determine the role of ovarian steroids on apoptosis and the atretic process in pigs.

### *Role of inhibin and activin*

Apart from the initial primary role of inhibin as an endocrine regulator of pituitary function and of activin as a stimulator of pituitary cell FSH secretion (de Jong, 1988), evidence from granulosa cell culture indicates that inhibin and activin, dimers of inhibin–activin $\alpha$ and $\beta$ subunits, act as intra-ovarian paracrine factors, regulating multiple facets of follicular development (Hillier, 1991; Findlay, 1994). Other studies have shown that increased expression of inhibin–activin subunits is associated with follicle growth during the early luteal phase of the oestrous cycle in pigs (Guthrie *et al.*, 1997; Garrett *et al.*, 2000). Multiple molecular forms of inhibin or the $\alpha$ subunit are detectable in follicular fluid of atretic and nonatretic follicles during the follicular and the early luteal phase of the oestrous cycle (Guthrie *et al.*, 1997). The high molecular mass forms of inhibin dimer (227, 121, 69 kDa) were more abundant in nonatretic follicles than in atretic follicles, indicating a higher rate of inhibin synthesis. Expression of $\alpha$ and $\beta$ subunit mRNAs, and cytochrome P450 aromatase protein increased by 102, 93 and 238%, respectively, as nonatretic follicles grew from the 1–2 mm into the 3–5 mm size class (Garrett *et al.*, 2000). In addition, expression of $\alpha$ and $\beta$ subunit mRNAs was positively correlated with the expression of a marker for cell proliferation, proliferation-associated nuclear antigen Ki-67 protein ($\alpha$: $r = 0.571$, $P = 0.0001$; and $\beta$: $r = 0.594$, $P = 0.0001$). In contrast, as follicle atresia increased, as indicated by granulosa cell apoptosis, from the nonatretic to the late atretic stage, the expression of $\alpha$ and $\beta$ subunit mRNAs, cytochrome P450 aromatase protein and cell proliferation-associated nuclear antigen Ki-67 protein decreased by 70, 83, 66 and 69%, respectively (Garrett *et al.*, 2000). These results indicate that granulosa cell proliferation and follicle growth are linked to the expression of inhibin–activin subunits. However, atresia was associated with a general decline in granulosa cell biosynthetic activity. In contrast, other findings indicate an atretogenic role for activin *in vivo* (Woodruff *et al.*, 1990) and *in vitro* (Ford and Howard, 1997). However, these results might also be explained by stimulation of androgen production by theca interna or by a blockade of terminal granulosa cell differentiation.

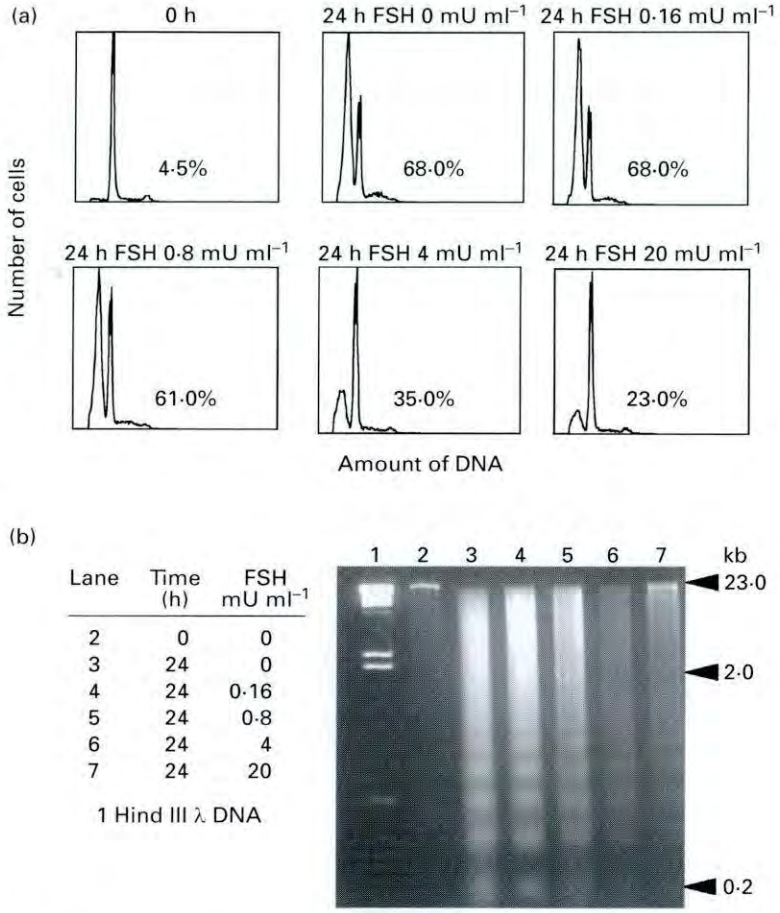


**Fig. 5.** Attenuating effect of FSH on apoptosis during 24 h culture of pig granulosa cells. (a) DNA histograms showing percentages of granulosa cells and (b) DNA internucleosomal cleavage showing low molecular weight DNA (< 23 kbp) fragments fractionated by agarose gel electrophoresis of DNA extracted from cells before culture (lane 2) and after culture (lanes 3–7). The absence of internucleosomal DNA fragments in lane 2 shows that the incidence of endogenous endonuclease activity was low before culture. DNA extracted from cells cultured for 24 h (lanes 3–7) shows an increase in DNA internucleosomal cleavage relative to noncultured cells and the attenuating effect of FSH (modified from Guthrie *et al.*, 1998).

### *FSH and growth factors* in vitro

Various growth factor pathways have been implicated in the growth, cell proliferation and differentiated cell functions of follicles (Hammond *et al.*, 1993). Culture of granulosa cells or follicles from untreated or equine chorionic gonadotrophin (eCG)-treated rats have been useful models for the study of granulosa cell apoptosis because the granulosa cells in these experimental models undergo spontaneous or culture-induced apoptosis (Tilly *et al.*, 1992b; Chun *et al.*, 1994). Gonadotrophins, EGF, transforming growth factor α, basic fibroblast

growth factor and IGF-I have been described as follicle survival or anti-apoptotic factors capable of suppressing granulosa cell DNA fragmentation in rats (Hsueh *et al.*, 1994).

Pig granulosa cells, like those of rats, undergo spontaneous or culture-induced apoptosis, and were used to test the hypothesis that FSH and IGF-I are anti-apoptotic, cell survival factors (Guthrie *et al.*, 1998). Representative DNA histograms of granulosa cells isolated from one pig (Fig. 5a) show that the percentage of apoptotic granulosa cells increased from 4.5% in freshly isolated cells to 68.0% after 24 h in culture. Apoptosis in granulosa cells decreased by half ($P < 0.01$) in the presence of FSH (4 NIH-S1 mU $ml^{-1}$) and reached a plateau between 4 and 20 FSH mU $ml^{-1}$. During the 24 h culture period, genomic DNA was cleaved into internucleosomal fragments forming 'DNA ladders' characteristic of deoxyribonuclease activity (Fig. 5b, lane 3). Internucleosomal cleavage was attenuated by 50% in the presence of FSH (Fig. 5b, lanes 6 and 7). IGF-I was also found to have an anti-apoptotic effect on granulosa cells (Guthrie *et al.*, 1998). Apoptosis decreased by 50% in cells subjected to 50–250 ng IGF-I $ml^{-1}$ compared with cells cultured for 24 h in the absence of IGF-I.

There are biologically significant differences in the regulation of apoptosis in isolated pig and rat granulosa cells. In rats, IGF-I and FSH did not attenuate apoptosis in cultured granulosa cells (Tilly *et al.*, 1992a; Hsueh *et al.*, 1994). In contrast, Guthrie *et al.* (1998) found that both FSH and IGF-I attenuated apoptosis in pig granulosa cells, but that EGF was not effective (H. D. Guthrie, unpublished). In rats, apoptosis in granulosa cells was attenuated only when FSH and IGF-I were added to the culture of intact preovulatory follicles (Tilly *et al.*, 1992a; Chun *et al.*, 1994). A review of several studies in rats and pigs indicates that apoptosis decreased in culture conditions that promoted granulosa cell proliferation (Guthrie and Garrett, 2000a).

### *The role of FSH* in vivo

Administration of charcoal-stripped pig follicular fluid induced changes in circulating concentrations of FSH in pigs indicating that FSH may play a critical role in maintaining a nonatretic population of follicles (Guthrie *et al.*, 1987, 1988). A decrease in circulating concentrations of FSH of 60% after treatment with charcoal-stripped pig follicular fluid (compared with charcoal-stripped pig serum) was associated with a reduction ($P < 0.05$) in total ovarian mass (by 39%) and of fluid volume (by 59%), and with a reduction ($P < 0.05$) in the number of 3–5 mm follicles from 29.6 to 2.9 per gilt (93% decrease). Follicles remaining after treatment with charcoal-stripped pig follicular fluid were opaque, which is indicative of atresia (Guthrie *et al.*, 1987). In another experiment, administration of FSH free of LH activity resulted in an increase ($P < 0.05$) in the number of 3–6 mm follicles compared with the saline control group (59.0 versus 30.8 per gilt, 48% increase) and restored the number of 3–6 mm follicles to that observed in the saline control group after charcoal-stripped pig follicular fluid treatment (0.2 versus 36.2 per gilt). There is a similar relationship between FSH secretion and the number of small and medium-sized follicles during the follicular phase and the periovulatory period of the next oestrous cycle. Selection and growth of the ovulatory cohort of follicles was accompanied by a 60–70% decrease in circulating concentrations of FSH and by atresia of non-ovulatory follicles and suspension of their replacement until after ovulation (Guthrie *et al.*, 1995; Guthrie and Garrett, 2000a). Regrowth of 3–5 mm follicles after ovulation is temporally related to increased circulating concentrations of FSH (Guthrie and Garrett, 2000a). During the cell differentiation required for the maturation of ovulatory follicles, FSH appears to become less important as these events progress in the presence of low concentrations of plasma FSH and granulosa cell FSH receptor. The critical factor for the survival of ovulatory follicles may be the greater LH-responsiveness of ovulatory follicles,

compared with non-ovulatory follicles, in terms of increased granulosa and theca cell LH receptor expression (Guthrie and Garrett, 2000a), LH binding and increased granulosa LH-sensitive adenylate cyclase activity (Channing *et al.*, 1982; Foxcroft and Hunter, 1985; Guthrie *et al.*, 1995; Guthrie and Garrett, 2000a). On the basis of the effects of FSH treatment *in vitro* and regulation of FSH secretion *in vivo*, we conclude that FSH plays a role in growth and survival of small and medium-sized follicles by stimulation of granulosa cell proliferation and attenuation of granulosa cell apoptosis.

### *Follicular IGF and IGFBP*

Although follicle atresia may be a result of decreased circulating concentrations of FSH and a reduction in the number of FSH receptors in the follicle population (Guthrie and Garrett, 2000a), an increase in the number of inhibitory IGF-I binding proteins (IGFBP) in granulosa cells may also play a role in biological neutralization of IGF-I (Hammond *et al.*, 1993). Analysis of follicular fluid from follicles recovered during preovulatory maturation and during the early luteal phase showed that the amount of follicular fluid IGFBP-2 present was three-fold greater in atretic than in nonatretic follicles (Guthrie *et al.*, 1995). The quantity of IGFBP-2 was positively correlated with the percentage of apoptotic granulosa cells per follicle, during preovulatory maturation ($r = 0.73$; $P \leqslant 0.001$) and during the early luteal phase ($r = 0.90$; $P \leqslant 0.001$; Fig. 4). Therefore, in several mammalian species, production or accumulation of IGFBP-2 may play a role in follicular atresia and granulosa cell apoptosis (Guthrie *et al.*, 1995). In studies of gene expression in relation to atresia and apoptosis, it is clear that follicular cell proliferation and atresia are regulated by a complex interaction between the granulosa, theca interna and factors originating from the systemic circulation.

### *Apoptotic pathway*

There is some evidence that caspase activity is associated with culture-induced apoptosis in pig granulosa cells (Guthrie *et al.*, 2000). The percentage of apoptotic granulosa cells increased ($P < 0.05$) from 2.3% in freshly harvested granulosa cells to 33% after 24 h in culture. The caspase inhibitor benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoro methylketone reduced apoptosis to 3%, which is similar to that in non-cultured cells. Reduction of granulosa cell apoptosis had no significant effect on steroidogenic activity during the culture period. Both APAF-1 and cytochrome *c* are expressed in cultured mouse granulosa cells and caspase 3 was activated during culture-induced apoptosis (Robles *et al.*, 1999). Further support for the role of caspases in ovarian apoptosis is that the expression of caspase 3 is associated with apoptosis in atretic follicles of rats (Flaws *et al.*, 1995). A new anti-apoptotic pathway, the IAP protein family, has been found in the ovaries of mice and rats. These proteins may play an important regulatory role in granulosa cell apoptosis and follicle atresia because in rats two IAPs, X-link inhibitor of apoptosis (XIAP) and human inhibitor of apoptosis protein 2 (HIAP-2), are localized in the cytoplasm and nuclei of granulosa and theca cells of healthy follicles and had decreased to low and non-detectable values in granulosa and theca cells, respectively, in atretic follicles (Li *et al.*, 1998). Another IAP, neuronal apoptosis inhibitory protein mRNA was strongly expressed in granulosa cells that were not undergoing apoptosis (Matsumoto *et al.*, 1999). A physiological role of IAPs is strongly supported by the observation that expression of neuronal apoptosis inhibitory protein was increased 2.4-fold after the administration of equine chorionic gonadotrophin (eCG) and hCG, a treatment known to inhibit granulosa cell apoptosis both *in vitro* and *in vivo*. Evidence for the operation of the positive induction pathway is also present in ovarian tissue with expression of Fas and the Fas ligand in atretic follicles of the rat (Hakuno *et al.*, 1996; Kim *et al.*, 1998).

Other signal transduction pathways may play a role in granulosa cell apoptosis and follicular atresia. An intense localization of angiotensin II receptor was found in atretic follicles of rats (Daud *et al.*, 1988). Expression of the angiotensin II receptor in granulosa cells may be inhibited by FSH in healthy follicles (Pucell *et al.*, 1988). If FSH support or FSH receptor function is lost, the angiotensin II receptor may be expressed and play a role in regulation of the atretic follicle. However, it remains to be established whether angiotensin II receptors are expressed as a result of atresia or whether expression of the receptors is involved in the mechanism of granulosa cell apoptosis. Although only limited studies have been conducted in pigs, clearly the physiologically regulated expression of the six major components of the apoptotic pathway are operating in the ovarian follicle and support the hypothesis that granulosa cell apoptosis is regulated through both negative and positive induction pathways.

## Conclusion

The rationale for the production of so many germ cells during early fetal life and their subsequent loss in pigs and other species is unknown. One explanation of the importance of apoptosis and follicular atresia is that oocytes may have a limited finite lifespan when they reach full size. Up to 70% of oocytes in 3–5 mm diameter follicles have initiated germinal vesicle breakdown (GVBD) in prepubertal (Day and Funahashi, 1996; Grupen *et al.*, 1997; H. D. Guthrie, unpublished) and post-pubertal gilts (Brüssow *et al.*, 1996; Guthrie and Garrett, 2000b). The incidence of GVBD did not differ significantly between atretic and nonatretic follicles before the start of ovulatory follicle maturation; however, follicles containing oocytes undergoing GVBD are eliminated before the preovulatory LH surge (Guthrie and Garrett, 2000b). Therefore, apoptosis and follicle atresia may be physiologically important as a barrier to ovulation of oocytes that have not remained in meiotic arrest during the period of rapid maturation of the ovulatory cohort.

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