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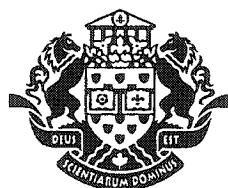
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Role and Regulation of X-Linked Inhibitor of Apoptosis Protein Expression
during Development of the Rat Ovarian Follicle *in vitro*

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Role and Regulation of X-Linked Inhibitor of Apoptosis Protein Expression during
Development of the Rat Ovarian Follicle *in vitro*

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To my parents,

Gan-Sheng Wang and Wei-Ping Sun

AUTHORIZATION

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CONTRIBUTION OF EACH CO-AUTHOR IN THE PUBLICATION

All studies reported in this thesis were conducted under the supervision of Dr. Benjamin K. Tsang. Unless otherwise specified all experimental work was carried out by Ms. Yifang Wang. All manuscripts reproduced in this thesis (Chapters 2, 3, and 4) were written by Ms. Yifang Wang and revised by Dr. Benjamin Tsang.

Chapter 2:

Title: Role and gonadotrophic regulation of X-linked inhibitor of apoptosis protein expression during rat ovarian follicular development *in vitro*

Journal: Biology of Reproduction 68: 610-619, 2003

Authors: Wang, Y., Rippstein P.U., and Tsang B.K.

Mr. Peter U. Rippstein (Head, Histology Laboratory, Department of Laboratory Medicine, The Ottawa Hospital, Ottawa, ON, Canada) contributed in the optimization in the sectioning of the ovarian follicles. Under his supervision, Ms. Dawn King and Ms. Nga Young (technologists of Histology Laboratory) embedded and sectioned all the follicles or ovaries used in the present studies. They also performed the Haematoxylin Phloxine Saffron staining on representative slides for morphologic examination.

Chapter 3:

Title: Involvement of transforming growth factor α in the regulation of rat ovarian X-linked inhibitor of apoptosis protein expression and follicular growth by follicle stimulating hormone.

Journal: Biology of Reproduction 66:1672-1680, 2002.

Authors: Wang, Y., Asselin E., Tsang B.K.

Postdoctoral fellow Dr. Eric Asselin synthesized the TGF α mRNA probes and advised on the *in situ* hybridization analysis.

Chapter 4:

Title: Involvement of inhibitory nuclear factor- κ B (NF κ B)-independent NF κ B activation in the gonadotropic regulation of X-linked inhibitor of apoptosis protein expression during ovarian follicular development *in vitro*.

Journal: Endocrinology 143: 2732-2740, 2002.

Authors: Wang, Y., Chan S., Tsang B.K.

Summer student Mr. Simon Chan worked under the supervision of Ms. Yifang Wang for two summer periods. He conducted a number of Western blots and assisted occasionally in cell culture reported in this chapter.

Chapter 5:

Title: Unpublished data

Section 5.1 Induction of Ovulation *in vitro*

Dr. Wai-sum O (Department of Anatomy, Hong Kong University, Hong Kong, China) initiated and collaborated on this section of study during her sabbatical period in Dr. Tsang's laboratory.

Section 5.4: Cyclic adenosine monophosphate (cAMP) increases XIAP protein levels in rat granulosa cells in a concentration-dependent manner.

Mr. Simon Chan performed most of the cell culture and Western blots included in this section during his training period as a summer student in Ottawa Health Research Institute. These results are included in this section.

ABSTRACT

Follicle stimulating hormone (FSH) is an important survival factor in the ovarian follicular development. The cellular and molecular mechanisms involved in the gonadotropic effect on follicular development and atresia have been extensively studied. Previous studies have demonstrated that a number of cell death and survival genes are regulated by gonadotropic stimulation or withdrawal during follicular development and atresia. Inhibitor of apoptosis proteins (IAPs) is a family of intracellular anti-apoptotic proteins. X-linked IAP (XIAP) has been shown to be involved in multiple biological activities (e.g. inhibition of caspase activities, promotion of ubiquitin-proteasome-mediated protein degradation, regulation of cell signaling pathways). Thus, it is a promising molecular target for the modulation of apoptosis and treatment of apoptosis-associated diseases. Although previous studies have demonstrated that FSH up-regulates ovarian follicular cell XIAP contents and suppresses apoptosis *in vivo*, whether these events are coincidental or causally related remains to be investigated. The present thesis research project examines: 1) the role and gonadotropic regulation of XIAP expression in rat granulosa cells during ovarian follicular development and atresia; 2) the possible involvement of intra-ovarian factors such as transforming growth factor alpha (TGF α) in the FSH-induced XIAP expression and follicular development; and 3) the signal pathways involved in the gonadotropic up-regulation of XIAP during follicular development *in vitro*.

A follicle culture system coupled to an adenoviral gene manipulation procedure has been established. FSH significantly increased follicular growth as evident by increases in follicular size, cell number and DNA contents *in vitro*. While cultured pre-antral or early-antral follicles showed a low XIAP content and evidence of apoptosis in the absence of FSH,

gonadotropin addition increased XIAP content and suppressed apoptosis. At low FSH concentration, adenoviral XIAP sense cDNA expression increased follicular cell XIAP and DNA contents, reduced apoptosis, and enhanced follicular growth, while XIAP antisense elicited opposite responses. FSH-induced XIAP up-regulation appeared mediated, in part, by the secretion and action of follicular TGF α . In cultured rat follicles, FSH-stimulated estradiol production, TGF α secretion, XIAP expression and follicular growth were suppressed by intra-follicular injection of a neutralizing anti-TGF α antibody or addition of the estradiol antagonist ICI 182780 to the culture media. These results support my hypothesis that the FSH induces follicular growth by stimulating granulosa cell proliferation via theca TGF α secretion and action in response to increased granulosa cell estradiol synthesis.

Since the promoter region of *XIAP* gene has nuclear kappa B (NF κ B) binding site, it is possible that the transcription of *XIAP* is mediated via NF κ B activation. FSH increased rat granulosa cell XIAP mRNA abundance and protein content. While the gonadotropin induced granulosa cell NF κ B translocation from cytoplasm to nucleus and increased NF κ B-DNA binding activity, pretreatment with an NF κ B translocation inhibitor suppressed FSH-stimulated XIAP expression. Unlike tumor necrosis factor alpha (TNF α), FSH failed to elicit a significant increase in granulosa cell phospho-I κ B and a decrease in total I κ B contents *in vitro*, and dominant negative I κ B expression effectively blocked the increase in NF κ B-DNA binding activity and XIAP protein content induced by TNF α but not by the gonadotropin. Granulosa cell phospho-Akt content was up-regulated by FSH, and the gonadotropin also increased NF κ B-DNA binding activity and XIAP content in granulosa cells, a response sensitive to the phosphatidylinositol 3-kinase (PI3K) inhibitors LY294002 and Wortmannin.

These findings demonstrate for the first time, that the FSH-induced XIAP expression is mediated through the NF κ B pathway via activation of PI3K rather than the classical I κ B kinase.

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LIST OF ABBREVIATIONS

Apaf-1	apoptotic protease activating factor 1
bFGF	basic fibroblast growth factor
Bcl	B cell chronic lymphocyte
BIR	baculovirus inhibitor of apoptosis repeat
BMP	bone morphogenetic protein
cAMP	cyclic adenosine monophosphate
CAD	caspase-activated deoxyribonuclease
CARD	caspase associated recruitment domain
CDDP	cis-platinum (II)-diamine-dichloride
CL	corpus luteum
CRE	cAMP response element
CREB	cAMP response element binding protein
db-cAMP	dibutyl-cyclic AMP
dd-ATP	dideoxy-Adenosine Triphosphate
DES	diethylstilbestrol
DIABLO	direct IAP binding protein with low pI
DISC	death-inducing signaling complex
DNA	deoxyribonucleic acid
dNTP	mixture of dATP, dTTP, dGTP and dCTP
eCG	equine chorionic gonadotropin
EGF	epithelial growth factor
ELISA	enzyme-linked immunosorbant assay
EMSA	electrophoretic mobility shift assay

ER	estrogen receptor
ERE	estrogen response element
FADD	fas-associated death domain
FAK	focal adhesion kinase
FasL	fas ligand
FITC	fluorescein isothiocyanate
FLICE	FADD-like ICE
FLIP	Fllice-like inhibitory protein
FSH	follicle stimulating hormone
GDF	growth differentiation factor
hCG	human chorionic gonadotropin
hOSE cells	human ovarian surface epithelial cells
HRP	horseradish peroxidase
i.p.	intraperitoneal
ICE	interleukin-1 β -converting enzyme
IgG	immunoglobulin G
ILK	integrin-linked kinase
IU	international unit
IVF	<i>in vitro</i> fertilization
JNK	c-Jun N-terminal kinase
KD	kilodalton
LH	luteinizing hormone

MAPK	mitogen-activated protein kinases
MMLV-RT	Moloney Murine Leukemia Virus reverse transcriptase
MOI	multiplicity of infection
NF κ B	nuclear factor kappa B
NIAMDD	National Institute of Arthritis, Metabolism & Digestive Disease
NIDDK	National Institute of Diabetes & Digestive & Kidney Disease
NLS	nuclear localization signal
PARP	poly (ADP-ribose) polymerase
PDK	phosphorylated by phosphoinositide-dependent kinase
Pfu	plaque forming unit
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol-3,4,5-trisphosphate
PKA	protein kinase A
PKB/Akt	protein kinase B
PKC	protein kinase C
PH	pleckstrin homology domain
PMSF	phenylmethylsulfonyl fluoride
PTEN	phosphatase and tensin homologue
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
s.c.	subcutaneous

SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SHIP	Src homology 2 (SH2)-containing inositol phosphatase
Smac	second mitochondria-derived activator of caspase
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween
TE	Tris-EDTA buffer
TGF α	transforming growth factor alpha
TGF β	transforming growth factor beta
TNFR	tumor necrosis factor receptor
TNF α	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	terminal transferase dUTP nick end labeling
UTRs	untranslated regions
UV	ultraviolet
VIP	Vasoactive intestinal peptide
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside
XAF1	XIAP-associated factor 1
α MEM	minimum essential medium alpha

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CHAPTER 1 GENERAL INTRODUCTION

1.1 THE OVARIAN FOLLICLE

1.1.1 Ovarian follicular anatomy and development

1.1.1.1 A life cycle of an ovarian follicle

The essential function of the ovary is to produce oocytes for fertilization and support pregnancy for successful propagation of the species. The ovaries of mature female mammals undergo regular cycles. In the life cycle of an ovarian follicle, a single primary oocyte serves as the basis for a primary follicle. Several primary follicles advance to the secondary follicle stage over several menstrual cycles. During each cycle, only dominant follicle(s) continues to mature under the influence of the follicle stimulating hormone (FSH) and reaches the Graafian stage. The luteinizing hormone (LH) surge at midcycle induces ovulation of the Graafian follicle. After ovulation, the remnant of the follicle becomes a corpus luteum. If pregnancy does not occur, the corpus luteum degenerates by a process called luteolysis, leaving a scarlike corpus albicans (Fig 1).

1.1.1.2 Follicular structure

The follicle is the basic functional unit of the ovary. A mature Graafian follicle is composed of theca cells, basement membrane, granulosa cells, antrum and oocyte (Fig. 1). In primary follicles, the granulosa cells secrete a viscous substance that forms the zona pellucida, a layer between the ovum and granulosa cells. An outer layer of thecal

Fig. 1: The life cycle of an ovarian follicle. Primordial follicles serve as the basis for primary follicles. Several primary follicles advance to the secondary follicle stage over several menstrual cycles. Usually only one (in human) or several (in rodents) follicles per cycle continues to mature under the influence of FSH and reaches the Graafian stage. Remaining follicles undergo atresia. A surge of LH at midcycle causes the Graafian follicle to ovulate. After ovulation, the remnant of the follicle becomes a corpus luteum. If pregnancy does not occur, the corpus luteum degenerates after about 12 to 14 days, leaving a scarlike corpus albicans (Adopted from Fig 6-1 p154 of Reproductive Endocrinology, 1999).

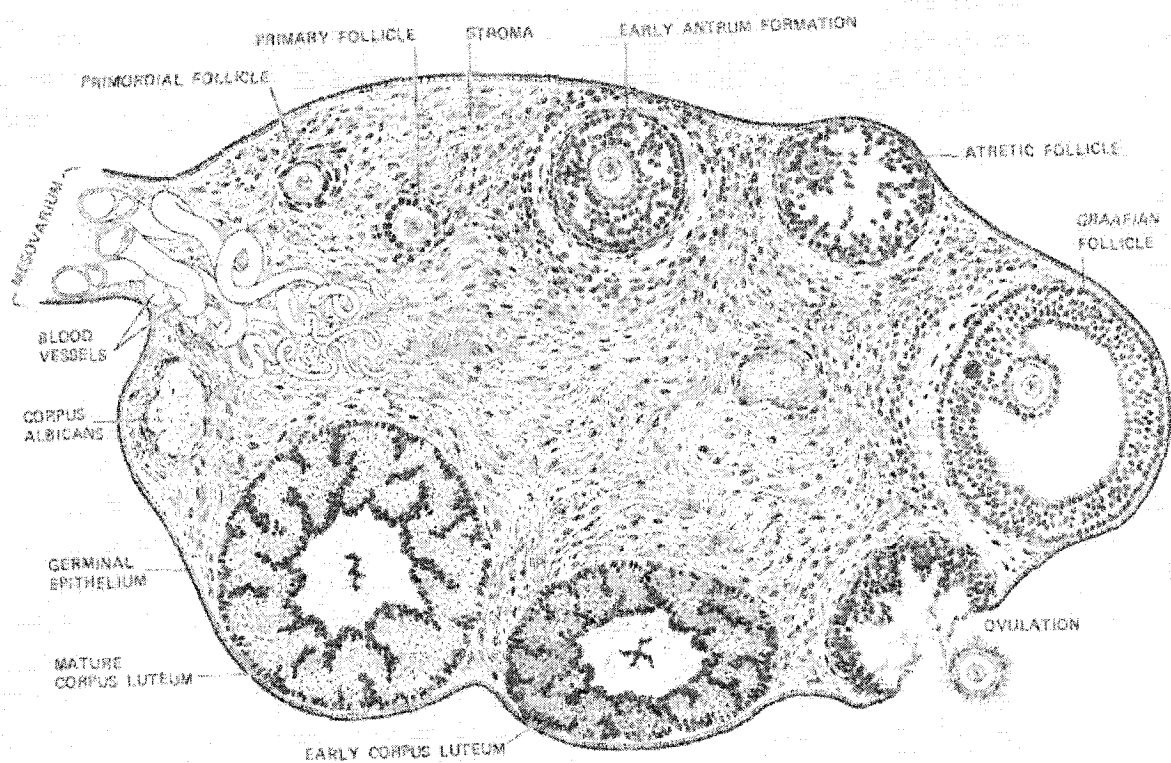


Fig 1

cells is added by differentiation of surrounding interstitial cells at secondary follicle stage. The theca cells form layers outside the basement membrane and are divided into the interna and externa. Theca cells produce the type IV collagen, fibronectin and laminin, which make up the basement membrane (Rajah and Sundaram 1994; Gore-Langton and Armstrong 1988; Lipner et al 1988). The theca interna has a vascular supply that forms a capillary network ending adjacent to the basement membrane, so that the granulosa layers, which do not have direct blood supplies, can get nutrients through the basement membrane. The outer layers of the follicle are composed of fibroblast and smooth muscle cells, together making up the theca externa. The formation of the antrum is facilitated by fluid secretion from the granulosa cells. The mural granulosa cells of the follicle are located just inside the theca layer, while other granulosa cells form the cumulus mass that supports the ovum. Both the granulosa and theca cell layers are functional as syncytia due to the presence of gap junctions between cells, which facilitate endocrine and paracrine signals to be spread rapidly and evenly through the population of granulosa or theca cells (Kidder and Mhawi 2002). It has been demonstrated that FSH increases connexin 43 expression at transcriptional level and stimulates granulosa cell communication through existing gap junctions (Kidder and Mhawi 2002; Sommersberg et al 2000; Granot and Dekel 1997; Granot et al 2000).

1.1.1.3 Follicular development

Follicular development is commonly divided into discrete stages. Primordial follicles become primary follicles when the enlarged oocyte is surrounded by a single layer of cuboidal granulosa cells. Multiple (3-5) granulosa cell layers resulted from

mitotic division, prior to the antral appearance, is the characteristic of the secondary follicle. In some species, theca cells are found around the basement membrane of secondary follicles. During the last phase of growth, fluid-filled cavity begins to appear between granulosa cells and eventually form a single large antral cavity. The appearance of a fluid-filled cavity marks the conversion of the secondary follicle into an antral follicle. In late stage, when follicle enlarges and acquires more fluid, the antral fluid becomes diluted and has similar protein content as found in serum (Yen and Adashi 1999). Mature follicles contain high concentrations of estrogen (1-2 $\mu\text{g/ml}$) in the follicular fluid due to its synthesis by granulosa cells and the presence of steroid binding proteins (Cigliano et al 2001). The accumulation of follicular fluid marks a period of rapid growth as the follicle increases from 200-300 μm to 700 μm in diameter within 2 days in the rat (Hirshfield 1991), or 1 mm to 12-16 mm in a week in the human (Macklon and Fauser 2001). By the end of this period, granulosa cells acquire receptors for LH and, along with the theca cells become competent to respond to the ovulatory surge of LH. Following ovulation, the basement membrane in the ruptured follicle breaks down and is infiltrated by blood vessels. The granulosa and theca cells in the ruptured follicle proliferate and fill the vacant antral cavity, transforming it into a corpus luteum (Yen et al 1999). Luteinization involves extensive hypertrophy of the granulosa and theca cells, and is accompanied by increased amount of cytoplasmic lipid droplets and estrogen and progesterone production for the conditioning of the uterus for implantation and nurturing a fertilized ovum.

1.1.2 Gonadotropins and follicular development

1.1.2.1 FSH on early stage of follicular development

The role of gonadotropins in early follicular development is controversial (Eppig 2001; Hohmann et al. 2001; Hsueh et al 2000; Roy and Greenwald 1986). Hypophysectomy (to remove the circulating gonadotropin) does not completely prevent the early development of follicles, indicating that gonadotropins are not critical for this process (Hirshfield 1991). Other evidence, however, suggests that gonadotropins are involved in the process of recruitment and early follicle development (Hsueh et al 2000; Hohmann et al. 2001). Intraperitoneal injection of eCG increases the number of primary and secondary follicles and variations in follicular growth initiation is correlated to the estrous cycle of the rat (Greenwald and Terranova 1988; Kishi and Greenwald 1999). When given during the early follicular phase (day 3 of menstrual cycle), FSH (at low dosages) can also induce multiple dominant follicle development in the human (Hohmann et al 2001). Moreover, hypophysectomy (pituitary removal) or suppression of gonadotropin by gonadotropin-releasing hormone (GnRH) antagonists results in reduced numbers of small growing follicles (Hirshfield 1991) and significantly more primordial follicles in the rat (Greenwald and Terranova 1988). Thus, gonadotropin appears to influence preantral follicular development but its role is unclear. This may involve intraovarian actions of steroids or growth factors, or growth regulatory signals produced by the oocyte. It is known that the oocyte regulates granulosa cell steroidogenesis (Vanderhyden and Tonary 1995; Vanderhyden et al 1993; Eppig 2001). Administration of estradiol to hypophysectomized rats is sufficient to produce large numbers of preantral follicles containing multiple layers of granulosa cells (Billig et al. 1993). Estrogen has

been shown to promote ovarian folliculogenesis and stimulate granulosa cell proliferation *in vivo* and differentiation *in vitro* and *in vivo* in rodents (Palter et al 2001).

1.1.2.2 FSH effects on granulosa cell proliferation and differentiation

The granulosa cells of preantral follicles are undifferentiated and mitotically active. The time required for each follicle to grow from one stage to another is dependent on the rate of granulosa cell proliferation. In rat, antrum formation begins when follicles grow beyond 300 μm in diameter. The highest rates of mitosis are observed prior to antrum formation, in follicles between 200-300 μm in size (Greenwald GS and Terranova 1988). Mitosis continues after the formation of the antrum, but the rate steadily declines as the follicle matures (Greenwald GS and Terranova 1988). Although the importance of FSH in the regulation of early follicle development remains controversial, this gonadotropin is known to be essential for the growth of follicles beyond the secondary stage of development (Macklon and Fauser 2001) and oocyte maturation (Patino et al 2001). Granulosa cell differentiation is indicated by increased gonadotropin receptor expression and steroidogenesis. After these granulosa cells have been exposed to gonadotropin, they acquire the ability to produce estradiol, follicular fluid and LH receptor (LHR) (Hirshfield 1991). Although there is the same amount of FSH exposure to every follicle, only granulosa cells in the dominant follicles appear to undergo FSH-induced differentiation. This indicates that FSH is a permissive signal for granulosa cell maturation, while the amount of FSH receptors (FSHR) on the granulosa cell determines its response.

1.1.2.3 Regulation of FSHR expression

In mammals, FSHR is found exclusively on granulosa cells from as early as the two-layer or primary stage of folliculogenesis. The discovery of different isoforms of FSHR, which result from hormone-induced receptor gene splicing (Babu et al 2001) raises the possibility that FSH action may be mediated through different receptors and signaling pathways. Up to four alternatively spliced transcripts have been described. An increase in steady-state levels of FSHR mRNA as well as a change in alternative splicing appear to be important during early folliculogenesis (Findlay and Drummond 1999).

1.1.2.4 Involvement of TGF β superfamily members in gonadotropic regulation of follicular development

The transforming growth factor β (TGF β) superfamily comprises a large group of growth and differentiation factors. They have been identified in a wide variety of species (from insects to mammals), and have been shown to have distinct but diverse growth and differentiative functions in many physiological systems. The most intensively studied members of this superfamily are TGF β s, activin/inhibin, and bone morphogenetic proteins (BMPs). These polypeptides have been reported to mediate numerous physiological processes including cell differentiation, tissue repair, bone formation, regulation of hormone secretion, immune response and various developmental functions in many organisms (Massague 1996; Tsuchida et al 1995).

Activin and inhibin were initially identified as a pair of antagonistic regulators of FSH production in the pituitary gland. Strong evidence has accumulated that activin and

inhibin are important regulating factors for many reproductive processes. Both inhibin and activin are local regulators of ovarian folliculogenesis. Granulosa cells have been identified as the source of inhibin and activin mRNA and protein in the ovary. Abundance of their mRNA changed throughout the follicular development, being maximal at secondary follicle stage (Findlay et al 2001).

FSHR expression is also regulated by oocyte-derived growth factors. For example, BMP-15 stimulates granulosa cell mitosis (proliferation) and inhibits FSHR mRNA expression (differentiation) in granulosa cells, thereby playing a critical role in the mechanism controlling ovarian folliculogenesis (Moore et al 2002). BMP-15, growth differentiation factor-9 (GDF-9) and BMP-6 are oocyte-derived members of the TGF β superfamily, and are known to promote the ovarian folliculogenesis (Otsuka et al 2001).

1.1.2.5 Effects of FSH on follicular maturation

It is clear that FSH is essential for the growth of follicles beyond the secondary stage of development (reviewed by Macklon and Fauser 2001). The granulosa cells in preantral follicles are essentially undifferentiated. They do not produce follicular fluid components, lack the ability to synthesize estradiol, and do not possess the receptor for LH. However, these cells express FSHR and, after exposure to the gonadotropin, acquire the ability to produce estradiol, follicular fluid and LHR. Granulosa cells are exposed to FSH throughout their development but only undergo FSH-induced differentiation once they have acquired the receptor and intracellular components necessary to respond to FSH. FSH initiates the final stages of follicular development, designed to make the

follicle capable of ovulation and luteinization in response to the LH surge. A variety of experimental inductions of luteinization emphasize the fact that it is the programming in the granulosa cells, rather than the nature of the inducing signal, that directs their final maturation (Hirshfield 1991; see a review by Richards 2001b).

1.1.3 Gonadotropins, steroidogenesis and follicular development

1.1.3.1 "Two-cell, two-gonadotropin" theory

The onset of antrum formation and steroidogenesis (Fig 2) are indicative of granulosa cell differentiation. Estrogen produced by the follicle exerts a negative feedback on FSH-release from the pituitary. In addition, estrogen has local effects in the follicle to induce FSHR. The combination of reduced circulating FSH level and increased local induction of FSHR results in follicles producing sufficient estrogen being selected as the "dominant" ones (Hsueh et al 1984). FSHR are only found on granulosa cells, while LHR are found on both granulosa cells of periovulatory follicles and on theca cells of all follicles. These two gonadotropins are the major regulatory hormones for steroid synthesis in the ovary and act on their respective target cells by activating adenylate cyclase-protein kinase A pathway (Gore-Langton and Armstrong 1988; reviewed by Hillier 2001). Estrogen is derived from androgens by the aromatization of testosterone by the aromatase, or by the aromatization and dehydrogenation of androstenedione. These precursors are derived from progesterone or pregnenolone, which is produced by

Fig 2: The "two-cell two-gonadotropin" regulation of steroidogenesis in the ovarian follicle in human. Cholesterol is predominantly acquired through the theca cell lipoprotein receptor (LDL-R), which binds and internalizes circulating cholesterol-lipoprotein complexes. Cholesterol, stored in lipid droplets, is esterified to fatty acids (FA). Binding of LH to its receptor stimulates cAMP production. This second messenger activates the de-esterification of cholesterol, its conversion into progesterone, and then androgen. Androgen leaves the theca and enters the granulosa cell, where it is converted to estrogen by the action of the aromatase. FSH binding to its receptor on the granulosa cell stimulates cAMP production, which induces the aromatase enzyme. Aromatase is also upregulated by estrogen and androgen. Granulosa cells can produce their own progesterone which can be converted to androgen in the theca. Both androgen and cAMP increase the production of progesterone by granulosa cells. Estrogen and cAMP both increase the sensitivity of granulosa cells to FSH by increasing the number of FSHRs. Estrogen can feedback on the theca cell to decrease the production of androgens. In more mature follicles, granulosa cells have LH receptors, which stimulate steroidogenesis by increasing cAMP production. The major pathway for the production of estrogen is through the conversion of cholesterol to androgen in the theca cell, and the subsequent aromatization of androgen in the granulosa cell. The conversion of cholesterol to progesterone is the rate-limiting step in androgen production, while the availability of androgen is the rate-limiting step in estrogen production. (Modified from Chapter 3 p78-80, Clinical Gynecologic Endocrinology and Infertility, Third edition. Leon Speroff, Robert H. Glass and Nathan G. Kase. Waverly Press, Inc.).

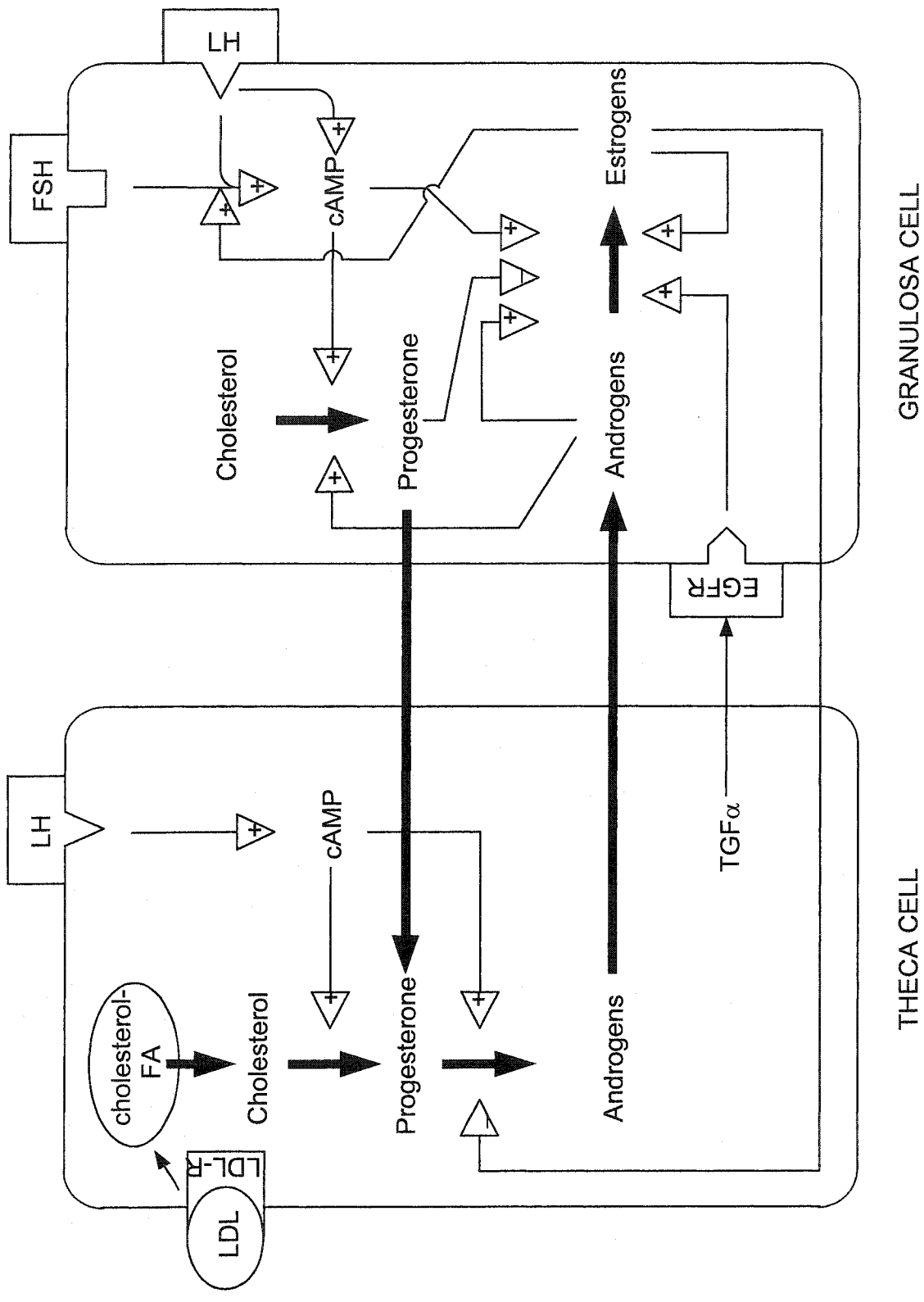


Fig 2

the action of P450 side-chain cleavage on cholesterol (Gore-Langton and Armstrong 1988). The major effect of FSH on granulosa cells early in follicular development is to induce aromatase activity, thus enabling the synthesis of estrogen by these cells. Later in follicular development, FSH also induces the activity of P450 side-chain cleavage enzyme (convert cholesterol to pregnenolone) in these cells to enhance the synthesis of progesterone. Granulosa cells can produce their own progesterone, which can be converted to androgen in the theca. Upon stimulation by LH, the main steroidogenic function of theca cells is the synthesis of androgens, which are the rate-limiting substrates for granulosa cell estrogen production (Gore-Langton and Armstrong 1988). Later in follicular development, both FSH and LH increase granulosa cell progesterone secretion by inducing the activity of P450 cholesterol side-chain cleavage enzyme and 3 β -hydroxysteroid dehydrogenase (3 β -HSD, convert pregnenolone to progesterone) (Hsueh et al 1984). Moreover, LH stimulates the production of progesterone and androgens by the theca cells (Ghersevich et al 2001). The expression of enzymes involved in progesterone and estrogen synthesis is often used as functional indices of granulosa cell differentiation. The combined action of FSH on granulosa cells and LH on theca cells is therefore necessary, throughout most of the development of the follicle, for the production of estrogens. This interaction is known as the "two-cell, two-gonadotropin" theory for the control of estrogen production (Gore-Langton and Armstrong 1988).

1.1.3.2 Steroid effects on follicular development

In addition to acting as a substrate for estrogen biosynthesis, androgens act on granulosa cells to enhance the activation of aromatase, as do estrogens, while progesterone inhibits aromatase. Both estrogen and androgen receptors are present in granulosa cells. Estrogens also have paracrine effects on theca cells to inhibit androgen production, thus acting as a potential negative feedback for estrogen production within the follicle (Fig. 2). Healthy follicles have high estrogen to androgen ratios, but this is reversed in atretic follicles (Greenwald and Terranova 1988; Gore-Langton and Armstrong 1988; Ghersevich S 2001; Reviewed by Nelson and Bulun 2001).

1.1.4 Intra-ovarian regulators of follicular development

It is clear that while gonadotropins are the principal hormones in the regulation of granulosa cell differentiation and follicle development, the effects of these hormones are influenced by a variety of intraovarian regulators, the production of which is regulated by gonadotropins.

1.1.4.1 Effects of growth factors

Epidermal growth factor (EGF) and transforming growth factor alpha (TGF α) are structurally related mitotic factors but have distinct single polypeptides chain (reviewed by Bridges 1999). Although encoded by different genes, these growth factors bind to the same receptor (Todaro et al 1990). EGF is a principal regulator of proliferation, differentiation and pattern formation in a variety of tissues and cell types (reviewed by

Carpenter and Cohen 1990; Cohen 1997). EGF and TGF α production in the ovary are regulated by gonadotropins (Roy and Greenwald 1991a & b; Kudlow et al 1987; Wang et al 2002a) and receptors for EGF (and also for TGF α) are known to be present in granulosa cells (Tekpetey et al 1995; Fujinaga et al 1994). Depending on prior hormonal exposure and the presence of other regulatory factors, EGF and TGF α can stimulate or inhibit proliferation or differentiation of granulosa cells *in vitro* (Leal et al 1990) as well as regulate the expression of their own receptors (Yang and Roy 2001). The proliferative activity of granulosa cells in a variety of species, including rat, human, hamster and hen, can be induced by EGF or TGF α (Gospodarowicz 1979; Lafrance et al 1993; Roy 1993; Wang et al 2002). TGF α , like EGF, is a potent inhibitor of gonadotropin-induced granulosa cell differentiation (Dodson and Schomberg 1987; Schomberg et al 1983) and anti-apoptotic factor (Wang et al 2002a). Both EGF and TGF α have been shown to inhibit estrogen and progesterone synthesis and LHR expression in rodent granulosa cells (Hsueh et al 1984; Mondschein and Schomberg 1984; Tilly et al 1992) and bovine oocyte meiotic progression (Sakaguchi et al 2002). Similarly, LH-induced granulosa cell progesterone production is also suppressed by EGF and TGF α (Tilly and Johnson 1990, Peddie et al 1994). The EGF receptor is a single polypeptide chain and the binding of EGF to the extracellular domain causes receptor dimerization (Boulougouris and Elder 2002) and autophosphorylation by EGF-R tyrosine kinase (Oksvold et al 2002). Autophosphorylation enhances the capacity of the receptor to phosphorylate intracellular effectors. The signal transduction pathways activated by EGF include the phosphatidylinositol pathway, which leads to activation of protein kinase C and an increase in intracellular Ca⁺⁺ concentration, and the Ras pathway, which leads to MAP

kinase activation (Boonstra et al 1995; Oksvold et al 2002). Similarly, basic fibroblast growth factor (bFGF) receptors are induced by FSH (Fujinaga et al 1994). bFGF suppresses granulosa cell aromatase expression and FSH-induced FSHR expression (Tilly et al 1992), as well as androgen synthesis in theca cells (Hurwitz et al 1990).

Another growth factor which has been intensively studied is Insulin-like growth factor (IGF-1). IGF-1 enhances gonadotropin-stimulated granulosa cell differentiation and is present at high levels in the ovary (Adashi 1998; Adashi 1995). FSH and estrogen stimulate IGF-1 production and the up-regulation of IGF-1 receptor mRNA (Hammond et al 1988). Growth hormone (GH) receptor has been identified in granulosa cells, and GH also increases the production of IGF-1 in the ovary (Davoren and Hsueh 1986).

1.1.4.2 Effect of cytokines

Cytokines and vasoactive intestinal peptide (VIP) are also important local regulators of ovarian cell differentiation (Karakji and Tsang 1995). Interleukin-6 (IL-6) is produced by granulosa cells in response to FSH (Gorospe and Spangelo 1993) and suppresses granulosa cell differentiation and steroidogenesis (Gorospe and Spangelo 1993; Hurwitz et al 1991). VIP acts through receptors on granulosa cells to elevate cAMP and increase estrogen production, especially in granulosa cells from immature follicles (Ahmed et al 1986).

1.1.5 Follicular atresia

1.1.5.1 Loss of germ cells by apoptosis

Formation of ovarian follicles begins during fetal development. In human, the first primordial germ cell is recognizable at 24 days after conception and proliferation of germ cells ends at 5 months and reaches a maximum of approximately seven million. However, five out of seven million are lost before birth, mainly by apoptosis. Germ cells are lost continuously after birth and only about 400 thousands remain in the ovary at the onset of puberty. Many follicles are recruited for development during each ovarian cycle. The process of follicular growth involves the recruitment, growth, ovulation and utilization of the follicle. However, only one (in human) and a few (in rodents) follicles become dominant and completely mature and ovulate. The remaining follicles undergo a degenerating process known as atresia. Therefore, only a small fraction of the potential pool of oocytes (about 400 follicles) are ovulated during the female reproductive life (Baker and Spears 1999).

Granulosa cells that have responded to FSH and undergone the process of differentiation become dependent on continued exposure to FSH. Preantral follicles less than 300 μm containing undifferentiated granulosa cells are rarely seen to undergo atresia (Hirshfield 1991; Hsueh et al 1994). However, the early penultimate stage of final follicular development (300-400 μm diameter follicles), when granulosa cells begin to differentiate and produce an antrum, is characterized by increased susceptibility to atresia (Hirshfield 1991; Hsueh et al 1994). FSH is present at low levels in immature rats but

does not increase in a cyclic pattern, which results in the atresia of all follicles at the early antral stage. Why follicles are particularly susceptible to atresia at this stage of development is not known. However, it is clear that, in mature cycling animals, only those follicles that begin the transition from the preantral to the antral state during the cyclic elevation of FSH will survive the transition period and have the opportunity to reach the preovulatory state. FSH induces the differentiation and progression of follicles toward maturity and ovulation, but most follicles die by the process of atresia. The onset of atresia has been characterized by the appearance of loosening layers and pyknotic nuclei in granulosa cells of the follicle. The number of pyknotic nuclei indicative of atresia has been estimated to be 5% and can be as high as 20% in the initial stages of the process (Byskov 1978; Hirshfield 1991).

1.1.5.2 Evidence of granulosa cell apoptosis

It has been established that follicular atresia occurs via apoptosis, and deoxyribonuclease I, a $\text{Ca}^{++}/\text{Mg}^{++}$ -dependent endonuclease is believed to be responsible for apoptotic DNA fragmentation detected in granulosa cell death (Zelevnik et al 1989; Boone et al 1995; Boone and Tsang 1997b). This theory is supported by ultrastructural and morphological studies of granulosa cells identifying characteristics of apoptosis, including membrane blebbing, chromatin condensation and cellular fragmentation and formation of apoptotic bodies (Hay et al 1976).

1.1.5.3 Influence of FSH on follicular atresia

In immature rats, FSH is present at low levels and does not increase in a cyclic pattern, thus resulting in the atresia of all follicles at the early antral stage. However, in mature cycling animals, only those follicles that begin the transition from the preantral to the antral state during the cyclic elevation of FSH will survive the transition period and have the opportunity to reach the preovulatory state. Although atresia in adult ovaries occurs at the transitional stage (i.e. the penultimate stage) between preantral follicle growth and the onset of antrum formation (Kim et al 1998), this process can also be induced at any stage of follicular development.

1.1.5.4 Time sequence of apoptosis in different cell types

Atresia in most adult ovaries occurs at the transitional stage between preantral follicle growth and the onset of antrum formation, but the process can be initiated at any stage of follicular development. In preantral follicles, the oocyte appears apoptotic first and then the cell death signal spreads to the granulosa cells (Greenwald and Terranova 1988; Eppig 2001). However, in more mature follicles, it is the granulosa cells that degenerate first while the oocyte appears to resist the onset of death before eventually regressing. In atretic follicles, theca cells die at the later stage compared to granulosa cells. Instead, they undergo hypertrophy and accumulate lipid droplets at the early stage of follicular degeneration. The theca and interstitial layer remains a source of androgen, which may contribute to the demise of granulosa cells in atretic follicles.

1.2 APOPTOSIS

1.2.1 Morphological and biochemical features of apoptosis

1.2.1.1 Morphological signs of apoptosis

Apoptosis is a physiological form of cell death with distinct morphological and biochemical characteristics (reviewed by Kerr 2002). Early morphological signs of apoptosis include the shrinkage of the cell and the loss of microvilli as the cell acquires a smooth exterior and dissociates from its neighboring cells. Cell shrinkage is accompanied morphologically by cytoplasmic and chromatin condensation, membrane blebs, formation of apoptotic bodies and phagocytosis by resident macrophages or neighboring cells (Arends et al 1990; Asselin et al 2000).

1.2.1.2 Biochemical changes

(1) DNA ladders

The morphological identification of apoptosis as a distinct form of cell death led to the search for biochemical events underlying these characteristic processes. One of the first discovered biochemical events associated with apoptosis was the degradation of genomic DNA into discrete fragments by $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease (such as DNase I, Boone et al 1995) or a caspase-activated deoxyribonuclease (CAD, Sakahira et al 1998). These low molecular weight DNA fragments are visible during electrophoresis as a 'ladder' pattern of 185 base pair multiples.

Initially, the degradation of DNA to form a ladder pattern on agarose gel electrophoreses was considered as a universal "biochemical hallmark" of apoptosis, but examples of programmed cell death without apparent DNA ladders suggested that DNA degradation might be a dispensable consequence of apoptosis, rather than a precipitating event (Saraste and Pulkki 2000; Ormerod et al 1994). DNA is also degraded into large molecular weight fragments (50 kbp) units, and no subsequent internucleosomal cleavage was detectable by conventional agarose gel electrophoresis. These results suggest that changes in the integrity of DNA indicative of the release of chromatin loop domains occur before cleavage at internucleosomal sites is initiated and that the latter is not an essential step in the apoptotic process (Oberhammer et al 1993a & b; Walker and Sikorska 1994; Rogalinska 2002).

2) Endonucleases

Ca²⁺/Mg²⁺-dependent endonuclease It was found that Ca²⁺/Mg²⁺-dependent endonuclease activity was present in thymocyte nuclei, and that this endogenous endonuclease was responsible for the DNA laddering during apoptosis. Studies on the role of these endonucleases in DNA degradation have led investigators to implicate known enzymes, such as deoxyribonuclease I (DNase I; Boone and Tsang 1997a; Mannherz et al 1995), and deoxyribonuclease II (DNase II; Barry and Eastman 1993), as well as some novel proteins (Pandey et al 1997) in the process of apoptosis. It has been demonstrated that DNase-I is responsible for apoptotic DNA fragmentation in ovarian granulosa and luteal cells (Boone and Tsang 1997a).

Caspase-activated deoxyribonuclease The functional CAD gene was localized to human chromosome 1p36.3 by fluorescence *in situ* hybridization. The CAD mRNA was expressed in a limited number of human tissues, including pancreas, spleen, prostate, and ovary. The expression of the CAD mRNA in human cell lines correlated with their ability to show DNA fragmentation during apoptosis. Overexpression of CAD potentiated DNA fragmentation by apoptotic stimuli in these cell lines, indicating that CAD is responsible for the apoptotic DNA degradation (Mukae et al 1998). Its inhibitor (ICAD) inhibits CAD-induced degradation of nuclear DNA and its DNase activity (Sakahira et al 1998).

1.2.2 Death signaling pathways

1.2.2.1 The role of caspases in apoptosis

A variety of aspartate-specific cysteine protease have been identified in the mammalian systems and grouped into a family of proteins called caspases (Alnemri 1997; Lesauskaite and Ivanoviene 2002), which cleave proteins after an aspartic acid residue. These enzymes have some homology to interleukin-1 β -converting enzyme, a mammalian cysteine protease (Yuan and Shaham et al 1993). Caspases are first synthesized as inactive proenzymes, and then activated by autocatalytic cleavage or the action of other proteases during the induction of apoptosis. Different substrates for distinct caspases have been identified as shown in Table 1 (Rosen and Casciola Rosen 1997). Caspase-3 plays a central role in the execution of the apoptotic program and is activated by the upstream caspases (eg. caspase-8, -9 and -12) in the cascade of proteolysis (Nagata 1997).

Table 1 Caspases and their substrates

New Names	Gene Map	Peptide Substrates	Auto-processing	Inhibited by XIAP	Substrates
Caspase 1	11q22	YEVD/X	Yes	No	Pro-IL-18; pro-IL-1b; Calpastatin; Bcl-Xlong
Caspase 2		VDVAD/X	Yes	No	
Caspase 3	4q34	DMQD/X	Yes	Yes	Ras; Calpastatin; p21; p27; Rb protein; ICAD; Bcl-Xlong; FLIP-L; Gelsolin; XIAP; PARP; DNA-PKcs; FAK; MEKK-1; Akt; Cytokeratin 18; mdm2; Ikb; NFkB; PKN; b-Catenin; Huntington; pro-IL-16; etc.
Caspase 4	11q22	LEVD/X	Yes		
Caspase 5	11q22	WVRD/S			
Caspase 6	4q25	DVVD/N	Yes	No	FAK; mdm2; Cytokeratin 18;
Caspase 7	10q25	DEV D/X	Yes	Yes	Calpastatin; FAK; mdm2; PARP;
Caspase 8	2q33	IETD/X	Yes	No	Cytokeratin 18; p27; p21-activated kinase 2; FLIP-L; Bid;
Caspase 9	1p34	LEHD/X	Yes	Yes	PARP;
Caspase 10	2q33	LEAD/X	Yes	No	FLIP-L;

1.2.2.2 Cell Death Pathways: death factors and their regulators

In almost all multicellular organisms, apoptosis appears to play an important role in the maintenance of cellular homeostasis. Apoptosis is tightly regulated by a set of genes that either promote apoptosis (via death factors) or stimulate cell survival (via survival factors). Recent discoveries have significantly advanced the understanding of biochemical and genetic requirements of distinct apoptosis pathways. There are three main pathways (Daniel 2000) leading to the activation of caspases: i.e. death receptor (caspase-8)-, mitochondria (caspase-9)- and endoplasmic reticulum (ER, caspase-12)-mediated apoptosis (Fig 3). The mature caspases (i.e. caspase-8, -9 and -12, respectively) then directly activate the downstream common execution caspase-3. In general, external stresses (UV, drugs etc.) result in activation of mitochondria, or ER-mediated cell death pathways and endogenous apoptosis triggers (TNF α , Fas Ligand etc.) stimulate surface receptor mediated cell death pathway (reviewed by Gupta 2003). There is evidence to suggest that, under certain conditions and in some cell types, these pathways may cross-talk to modulate or promote each other's signaling activity and function (reviewed by Gupta 2003).

(1) Mitochondria-mediated apoptosis:

Mitochondria hold the secrets of life and death. While they produce metabolic energy in the form of ATP, they are involved in the process of apoptosis and harbour proteins that are potentially lethal to cells. These proteins are found in the intermembrane space, the space between the outer and inner mitochondrial membranes. One of such

Fig 3 Three classical pathways of caspase-mediated apoptosis.

Death receptor [Fas and TNF α receptor (TNFR)]-, mitochondria- and endoplasmic reticulum (ER)-mediated apoptosis are three main mechanisms in caspase-mediated apoptosis. Exposure of the cell to Fas Ligand or TNF α or is associated with activation of receptor death domains (FADD and TRADD, respectively) and of caspase-8. Alternatively, some external stresses (such as UV, drugs etc.) result in activation of mitochondria and/or ER-mediated cell death pathways. Activation of mitochondria-mediated death pathway leads to mitochondrial cytochrome c (cyt c) release into cytoplasm and its binding to the cytosolic factor apoptotic protease activating factor-1 (Apaf-1) to form apoptosome. The mitochondria-dependent apoptosis can also induced by the cleavage of, release of cytochrome c, formation of apoptosomes. Cleavage of Bid by caspase-8 and subsequent activation of Bax causes the cross-talk between death receptor- and mitochondria-mediated apoptosis pathways. In ER-mediated apoptosis, microsomal caspase-12 is activated. The mature caspases (i.e. caspase-8, -9 and -12, respectively) then activate the common downstream execution caspase-3 and induce cell apoptosis.

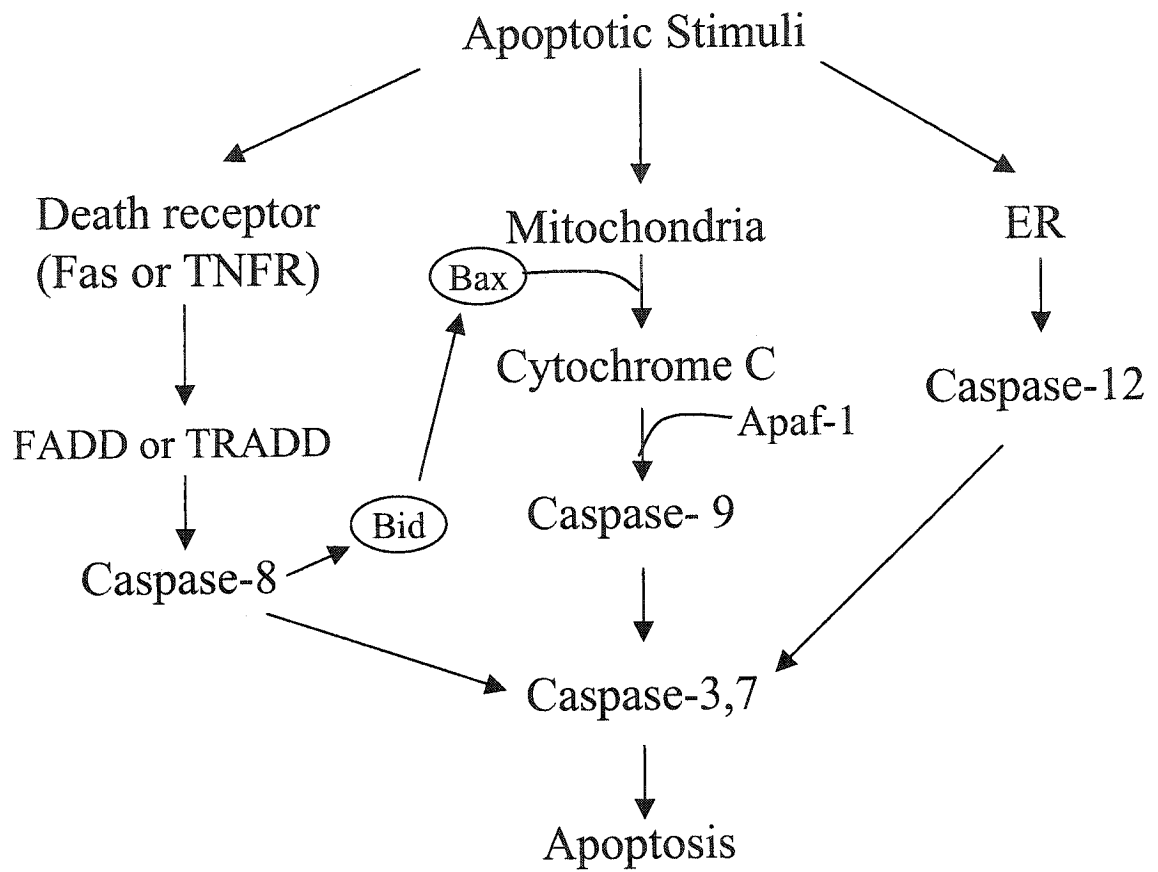


Fig 3

protein is cytochrome c, an essential component of the respiratory electron transport chain, but also a potent activator of apoptosis when released into the cytosol (Jia et al 2001; Alnemri 1999).

Mitochondria-mediated cell death pathway is activated by many triggers, such as growth factor deprivation, glucocorticoid, cytotoxic agents and mitogenic oncogenes. These stimuli lead to an increase in the permeability of the outer mitochondrial membrane and promote the release of cytochrome c from mitochondria into cytoplasm. Once in the cytoplasm, cytochrome c binds to a cytosolic factor apoptotic protease activating factor-1 (Apaf-1) and forms the apoptosome (reviewed by Cecconi and Gruss 2001; Cain et al 2002). The complex then recruits and activates procaspase-9, which subsequently activates procaspase-3. The observation that apoptosis is suppressed in caspase-9- or Apaf-1-deficient mice indicates that caspase-9 (Kuida et al 1998; Hakem et al 1998) and Apaf-1 (Matsuki et al 2002; Yoshida et al 1998) are important players in this process. Bik and Bid, proapoptotic members of the Bcl-2 family, are cell death triggers. They are known to activate Bax and subsequently stimulate mitochondrial cytochrome c release and subsequently caspase-9 activation *in vitro* (Lim et al 2002). However, Bax can induce mitochondrial damage and cell death even when caspases are inactivated (Jurgensmeier et al 1998), suggesting that existence of caspase-independent but mitochondria-mediated cell death pathway (Kroemer 1999).

Using immunochemical and immunogold electron-microscopic techniques, procaspase-9 has been shown to be located in the mitochondrial intermembrane space in a variety of cell types, upon induction of apoptosis, mitochondrial pro-caspase-9 translocates to the cytosol and to the nucleus (Costantini and Bruey et al 2002). This

phenomenon is inhibited by transfection with Bcl-2. This Bcl-2-mediated regulation of mitochondrial membrane permeabilization may contribute to apoptosis control by preventing mitochondrial procaspase-9 from interacting with its cytosolic activators. The mechanism by which caspase-9 enters the nucleus is currently unclear. Although caspase-9 has been considered as an initiator caspase, the observation of caspase-9 translocation from mitochondria to the nucleus during neuronal apoptosis suggests that it may also cleave nuclear targets and would therefore also have an executioner role in neurons (Cowan et al 2001; Roth and D'Sa 2001).

(2) Death receptor-mediated apoptosis:

(a) Death receptor and ligands

Fas Ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL/APO-2L) and TNF- α are key inducers in surface receptor-mediated cell death pathways and will be reviewed hereafter. The ligation of death receptors, including Fas, DR (death receptor) and the tumor necrosis factor receptor (TNFR), results in death domain-mediated activation of procaspase-8 and procaspase-3.

(b) Fas/FasL pathway

Mechanisms of Fas/FasL-based apoptosis

The Fas (APO-1/CD95) receptor, a member of the TNF/nerve growth factor (NGF) receptor superfamily, is a 45-kD transmembrane protein known to induce apoptosis following binding with Fas, a 31-kD type II membrane protein with its C-terminal located in extracellular domain. Fas trimerizes upon ligation and a signal to

enter the apoptotic pathway is transmitted via its intracytoplasmic death domain (FADD) for the activation of pro-caspase-8 and 3 (Schneider and Tschopp 2000).

Fas is expressed in most cells, whereas FasL is predominantly expressed in inflammatory cells such as macrophages and activated T-cells (Shimizu et al 2002). In the immune system, although mature T-cells constitutively express Fas, activation by antigens up-regulates this expression and makes the T cells sensitive to Fas-mediated apoptosis. As demonstrated in Fas-based immune down-regulation at the lymphocyte level (Fig 4), there are three possible mechanisms involved in Fas-mediated apoptosis (Nagata and Golstein 1995): 1) Fas and FasL are expressed on different cells, leading to Fas-based cell death through a “trans” mechanism; 2) Upon T-cell receptor activation or reactivation, both Fas and FasL appear on the same cell. These molecules, situated on the cell surface, interact in an unknown manner, possibly by membrane folding and result in cell death by a “cis” mechanism; 3) Soluble FasL released from an activated lymphocyte can undergo apoptosis by “cis” or “trans” mechanism, that is, either on the same or on other Fas-bearing lymphocytes. The soluble FasL (sFasL) is generated from membrane FasL being cleaved off by matrix metalloproteinase-7 (MMP-7). Both mFasL and sFasL are functional and can induce caspase-8-mediated cell death, although sFasL to a lesser extent. Recent studies have shown that an excess of sFas is the key cause of some diseases (Shimizu et al 2002). Some evidence indicates that FasL contributes to immune privilege in tissues and tumors, whereas other data demonstrates that FasL can elicit inflammation. These results suggest that the function of FasL differs from system to system and species to species (Nagata 1998). For example, human soluble FasL but not

Fig.4. A schematic representation of three mechanisms that may be involved in Fas-based immune down-regulation at the lymphocyte level. (A) Fas and its ligand, FasL, may be expressed on different cells, leading to Fas-based death in trans through a cytotoxicity-like mechanism. (B) “Suicide” in cis. Upon T-cell receptor-transduced activation or reactivation, both Fas and FasL appear on the same cell, which is then induced to die. These molecules, situated at the cell surface, interact in an unknown manner, possibly by membrane folding. (C) Soluble FasL released from an activated lymphocyte can act in cis or trans, that is, on the same or on other Fas-bearing lymphocytes. APC: antigen-presenting cell. (Adopted from Nagata and Golstein Science 1995; Vol 267, p1452).

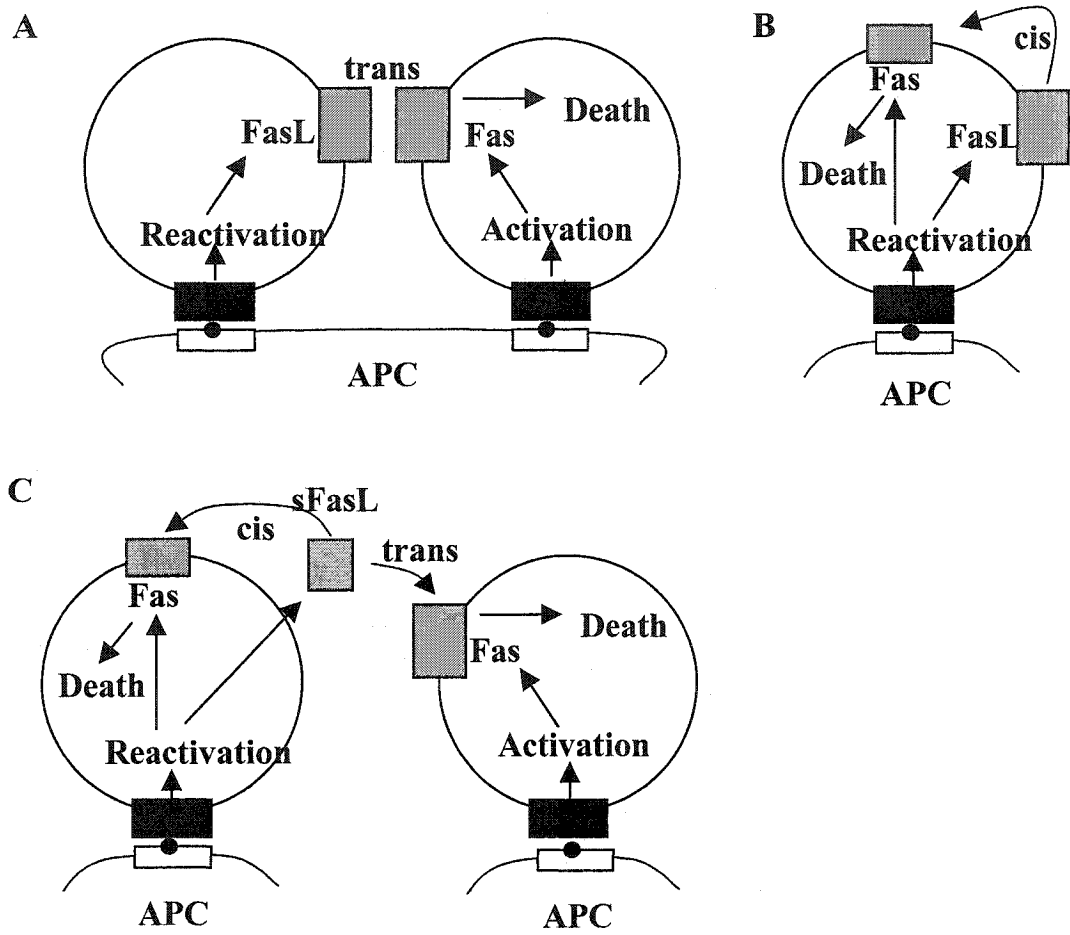


Fig 4

murine FasL has apoptotic activity, and the physiological function of sFasL in human, but not in mice, is to delete the potentially auto-reactive "memory" lymphocytes (Kim et al 2002a).

(c) TNF- α -induced cell death

Tumor necrosis factor- α (TNF- α) is a pro-apoptotic factor and induces differentiation and proliferation of many cell types (Terranova 1997). The balance between pro-apoptotic and anti-apoptotic intracellular factors induced by TNF α determines the final fate of the cell (death or survival). The actions of TNF α are mediated by its two receptors, TNFR1 and TNFR2. TNFR1 contains an intracellular death domain (TRADD) required for induction of apoptosis and is also coupled to an NF κ B pathway. Binding of TNF α to its receptors activates caspase-8 and caspase-3 as well as induces I κ B phosphorylation and degradation, and subsequently activates NF κ B. NF κ B activation regulates the expression of a number of genes involved in the prevention of TNF α -induced apoptosis, such as zinc finger protein A20 (Kawanishi 2000), some members of the Bcl-2 family (Zong et al 1999), Flice-like inhibitory protein (FLIP, Xiao et al 2002; Xiao et al 2003), and inhibitor of apoptosis proteins (IAP) (Xiao et al 2001). TNF α has also been shown to increase murine granulosa cell expression of Fas and Fas ligand and to induce Fas-mediated apoptosis (Quirk et al 1998).

(d) TRAIL/APO-2L-induced cell death

TRAIL is a member of the TNF family and promotes apoptosis by binding to the transmembrane receptors TRAIL-R1/DR4 and TRAIL-R2/DR5. Its cytotoxic activity is

relatively selective to the human tumor cell lines without much effect on normal cells. Hence, it exerts an anti-tumor activity without causing toxicity, as apparent by studies with several xenograft models (reviewed by Srivastava 2001). The major pathway of its action proceeds through the formation of death-inducing signaling complex (DISC) and activation of caspase-8. The apoptotic processes, therefore, follow two signaling pathways: (a) mitochondria-independent activation of caspase-3, and (b) mitochondria-dependent apoptosis resulting from cleavage of BID by caspase-8, subsequently activation of BAX, release of cytochrome c, formation of apoptosomes (complex of Apaf-1 and cytochrome c, and activation of caspase-9 and the downstream caspases. Bcl-2 and Bcl-X_L have no effect on TRAIL-induced apoptosis in lymphoid cells (mitochondria-independent), whereas these proteins block or delay apoptosis in non-lymphoid cancer cells (mitochondria-dependent), suggesting Bcl family of proteins are important survival factors in mitochondria-dependent death pathway (Srivastava 2001).

(3) Endoplasmic reticulum (ER)-mediated apoptosis

In endoplasmic reticulum-mediated apoptosis, activation of microsomal caspase-12 by the endoplasmic reticulum stress response appears required to activate downstream caspases (Daniel 2000; Morishima et al 2002). Different apoptotic stimuli result in activation of different cell death pathways and cross-talk between some pathways. As reported by Rao et al (2002), while Apaf-1^{-/-} (null) fibroblasts were resistant to cell death initiated by proapoptotic stimuli such as tamoxifen, they were susceptible to apoptosis induced by thapsigargin and brefeldin-A, although all three induce ER stress. These results indicate that ER stress-induced programmed cell death can be Apaf-

1/mitochondria cell death pathway activation-dependent (in response to tamoxifen) or – independent (thapsigargin and brefeldin-A). There is no cross-talk between ER-pathway and the cell surface receptor pathway, since the ER pathway could be inhibited by catalytic mutants of caspase-12 or/and caspase-9 and but not of caspase-8. Thus, the end result of either pathway appears to be caspase activation and the cleavage of specific cellular substrates, resulting in the morphological and biochemical changes associated with the apoptotic phenotype (reviewed by Zimmermann et al 2001).

(4) Role of p53

The p53, an anti-proliferative transcription factor, enhances the transcription rate of several genes believed to modulate both mitosis and apoptosis. This protein plays a critical role in regulating cell proliferation and death, and is believed to be involved in cell cycle arrest during the G1 to S phase transition, DNA repair and in the control of genome integrity (Levine 1997).

1.2.2.3 Cross-talk among cell death pathways

Similar to the cross-talk between mitochondria- and ER-mediated pathways as mentioned in last section [1.2.2.2 (3)], cross-talk also exists between apoptosis pathways mediated by mitochondria and cell death receptors (Fig 3).

The pro-apoptotic Bcl-2-family (B cell chronic lymphocyte: Bcl) member Bid mediates a mitochondrial amplification loop essential for the induction of apoptosis in selected cells. Bid participates in the apoptotic pathway by inducing cytochrome c release

from mitochondria in response to TNFR1/Fas signaling. Bid translocates from the cytoplasm to the outer mitochondrial membrane upon its cleavage and activation by the caspase-8, leading to activation of Bax, disruption of the mitochondrial outer membrane and the release of mitochondrial apoptogenic factors, including cytochrome c, Apaf-1 and procaspase-9 (Van Cruchten and Van Den 2002).

Studies with Bid-knockout animals have confirmed that Bid is essential for the cross-talk between death receptor- and mitochondria-mediated pathways. When Bid-deficient mice are injected with an activating antibody directed against Fas, they nearly all survive, whereas wild-type mice die from hepatocellular apoptosis and haemorrhagic necrosis (Yin et al 1999). About half of the Bid-deficient animals had no apparent liver injury and showed no evidence of activation of caspase-3 and -7, although the initiator caspase-8 was activated. Other Bid-deficient mice survived with only moderate damage: all three caspases (caspase-8, -3 and -7) were activated but their cell nuclei were intact and no mitochondrial cytochrome c was released.

1.2.3 Cell survival genes

Cancers do not necessarily arise as a result of an increased rate of cell proliferation. Rather, it is the critical balance between the rate of cell cycle progression (cell division) and cell growth (cell mass) on one hand, and apoptosis on the other, that is important. It has been well established that the induction of apoptosis is associated with not only the expression of specific “death” genes as discussed above, but also the down-regulation of “survival” factors (such as Bcl-2, FLIPs and IAPs). In this section, a few key cell survival factors and their roles in the regulation of apoptosis will be discussed.

1.2.3.1 Bcl-2 related cell survival gene products

An expanding family Bcl-2 proteins plays an important role in the decision step of apoptosis, and the differential expression of Bcl-2 members and their binding proteins allows the regulation of apoptosis in a tissue-specific manner mediated by extra- and intracellular signals (Hsu and Hsueh 2000). Unlike other oncogenes, Bcl-2 does not induce proliferation, but instead prevents cell death (Reed 1994). Bcl-2 family proteins are mitochondrial membrane proteins and are key regulators of the mitochondria-mediated cell death pathway. Recently, localization of Bcl-2 in the nuclear envelope and endoplasmic reticulum has also been demonstrated (Krajewski et al 1993; Daniel 2000). Cellular stress induces pro-apoptotic Bcl-2 family homologues, such as Bid, to translocate from the cytosol to the mitochondria, where they induce the release of cytochrome-c by mitochondria membrane transformation. While the anti-apoptotic Bcl-2 or Bcl-X_L enhances Ca^{2+} buffering capacity, it inhibits cytochrome c release, thereby preserving cell survival (Reed and Green 2002; Green and Reed 1998). Apoptosis induced in a variety of cell types by chemotherapeutic drugs, ionizing radiation, hydrogen peroxide, growth factor deprivation, calcium, TNF- α , and Fas activation are blocked by Bcl-2 (Reed 1994; Oltvai and Korsmeyer 1994). In addition, Bcl-2 blocks early morphological and biochemical events of apoptosis (Oltvai et al 1993).

1.2.3.2 Flice-like inhibitory protein (FLIP)

FLIP is a FADD-binding suppressor of apoptosis. FLIP is present in long (FLIP_L) and short (FLIP_S) isoforms. Both isoforms contain two death effector domains (DED), a

structure resembling the N-terminal half of caspase-8. Through DED-DED interactions, FLIP functions as a dominant negative for FLICE and blocks Fas-mediated apoptosis by preventing the activation of caspase-8. Furthermore, FLIP over-expression blocks TNF α -induced cell apoptosis in the presence of cycloheximide (Wu et al 2002). However, the role of FLIP is controversial in some cell types as its over-expression has been reported to induce apoptosis (reviewed by Tschopp et al 1998).

1.2.3.3 The role of inhibitor of apoptosis proteins

The inhibitor of apoptosis proteins (IAPs) is a family of intracellular anti-apoptotic proteins. The initial discovery of IAPs in baculoviruses was soon followed by their identification in metazoan species including *Drosophila*, *C. elegans*, yeast and vertebrates (reviewed in LaCasse et al 1998; Deveraux and Reed 1999). IAPs are widely expressed from both phylogenic and physiologic points of view. The diversity of triggers against which the IAPs suppress apoptosis is greater than that observed for any other family of apoptosis inhibitors, including Bcl-2 family. The IAP family includes X-linked IAP (XIAP, cIAP-3), human IAP-1 (HIAP-1, cIAP-2), human IAP-2 (HIAP-2, cIAP-1), neuronal apoptosis inhibitory protein (NAIP), Survivin, Livin (Fig 5) (Yang and Li 2000; Miller 1999 LaCasse et al 1998). IAPs are characterized by the presence of a caspase recruitment domain and an N-terminal BIR motif, that includes a conserved cysteine and histidine core sequence Cx₂Cx~Wx₃Dx₅Hx₆C. The number of BIR domains in IAPs is variable, ranging from one to three; they are, however, invariably present in the N-terminal half of the protein and are essential for the anti-apoptotic activity of IAPs by virtue of their ability to bind and inhibit distinct caspases. At least four IAP members

Fig. 5 Diagrammatic representation of the structure of mammalian IAP family members. All IAPs have one or more baculovirus inhibitor of apoptosis repeat (BIR) motifs required for biological activity. Apart from Naip and Survivin, they have RING Zing finger involved in protein-protein interactions and auto-ubiquitination. Hiap-1 and Hiap-2 also have caspase recruitment domain (CARD).

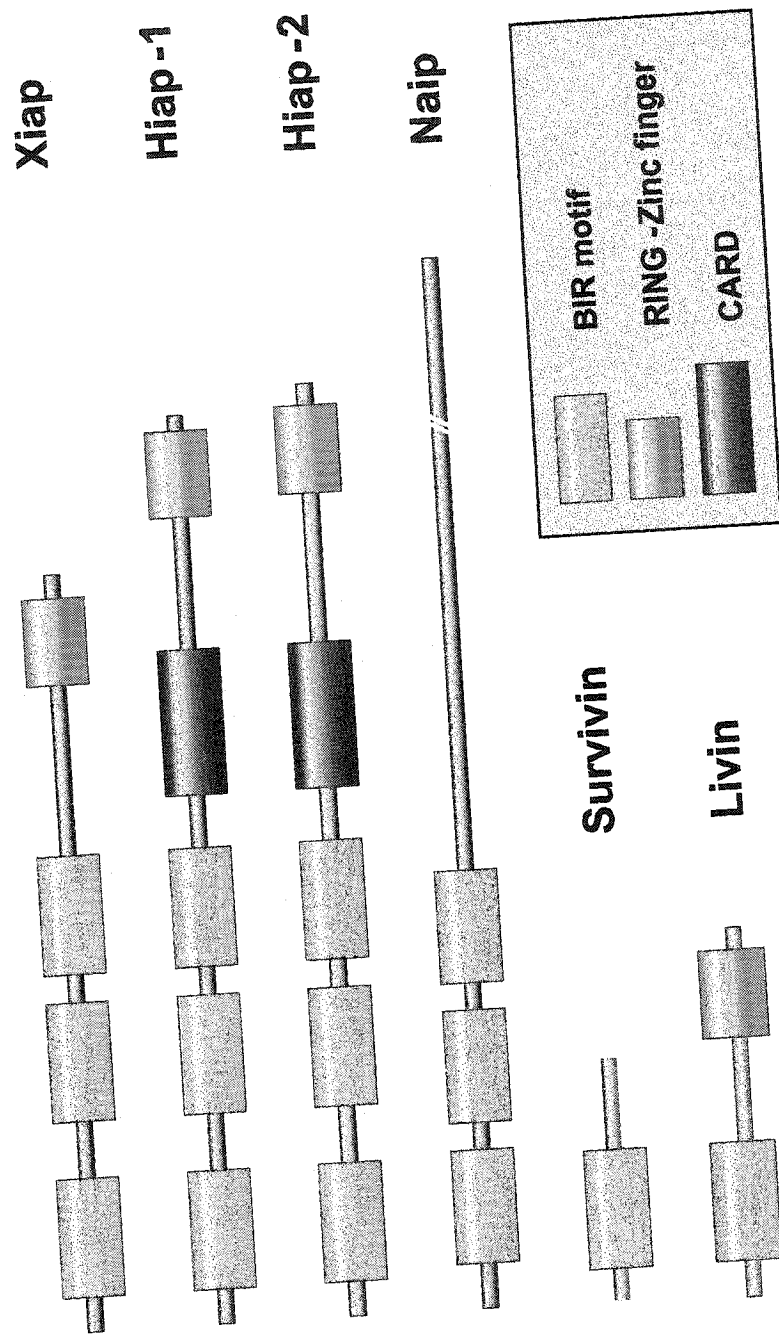


Fig 5

have been reported to directly inhibit caspases (Budihardjo et al 1999). Human XIAP (Deveraux et al 1997), cIAP-1, and cIAP-2 (Roy et al 1997; Deveraux et al 1998) can inhibit caspase-3, -7 and -9, but not caspase-1, -6, or -8. Livin shows weak inhibition of caspase-3, but more robust inhibition of caspase-9 (Kasof and Gomes 2001). With the exception of NAIP and Survivin, the IAPs also contain a C-terminal RING-Zinc finger domain believed to be required for protein-protein interactions. Recently studies have shown IAPs have RING-Zinc finger-dependent ubiquitin ligase activity. XIAP auto-ubiquitination has been demonstrated as a major degradation pathway in dexamethasone-treated thymocytes (Yang and Li 2000).

The central mechanisms of IAP apoptotic suppression appear to be through the direct inhibition of caspase activities (primarily caspase 3, 7 and 9) and modulation of the transcription factor, nuclear factor kappa B (NF κ B). Although evidence for a direct oncogenic role for the IAPs has yet to be delineated, some studies point towards this class of protein playing a role in oncogenesis. The strongest evidence for IAP involvement in cancer is shown in Survivin. Survivin has several unique characteristics that differ from related IAP members. Unlike most of the IAP proteins, which contain two to three BIR motifs, Survivin contains only a single BIR domain with an α -helical coiled-coil domain, which replaces the canonical RING finger found in the COOH-terminus of cIAP1, cIAP2 and XIAP (Chantalat et al 2000). Due to the combination of these features, Survivin is able to regulate both cell proliferation and apoptotic cell death. Survivin is hypothesized to inhibit a default apoptotic cascade initiated during mitosis via the α -helix coiled-coil domain binding to mitotic spindle microtubules (Li et al 1999). Survivin is expressed in most transformed cell lines and cancers tested to date in a cell-cycle-dependent manner,

with highest levels found during the G2/M phase of the cycle (Li et al 1998a; LaCasse et al 1998). Survivin has been shown to inhibit caspase directly and apoptosis in general (Kawamura et al 2003).

XIAP has been shown to be a direct inhibitor of caspase-3 and caspase-7 and also to modulate the Bax/cytochrome c pathway by inhibiting caspase-9 (Bratton et al 2002). Over-expression of XIAP has been shown to protect Chinese Hamster Ovary (CHO) and RAT-1 cells from menadione-mediated or growth factor withdraw-mediated apoptosis. It also suppressed the apoptosis in HeLa cells induced by transient transfection with interleukin-1 β converting enzyme and Sindbis virus (Sauerwald et al 2002). The second line of evidence for the involvement of IAP in cancer comes from their emerging role as mediators and regulators of the anti-apoptotic activity of v-Rel and NF κ B transcription factor families.

XIAP expression can be regulated at both transcription and translation levels. Since *XIAP* contains a NF κ B binding motif in exon specifying as the 5'-UTR of its mRNA and XIAP protein is a well-established NF κ B-dependent cell survival factor, its mRNA level maybe regulated by the NF κ B pathway. XIAP mRNA is also translated by a rare cap-independent mechanism mediated by a specific sequence called IRES (Internal Ribosome Entry Site), which is found in the XIAP 5' UTR (reviewed by Holcik et al 2001). However, IRES-dependent translation is only existing when cells under acute stresses.

The caspase-inhibiting activity of XIAP is negatively regulated by at least three intracellular XIAP-interacting proteins: XIAP-associated factor 1 (XAF1), Smac/DIABLO and HtrA2/Omi (Liston et al 2001; Srinivasula et al 2000; Du et al 2000;

Verhagen et al 2000; Suzuki et al 2001a). In contrast to XIAP, which is found primarily in the cytoplasm, XAF1 is a nuclear protein, and was identified in a yeast two-hybrid screen based on its ability to bind XIAP. XAF1 was found to antagonize the ability of XIAP to suppress caspase activity and cell death *in vitro*. Over-expression of XAF1 triggers a redistribution of either endogenous or over-expressed XIAP from the cytosol to the nucleus to neutralize XIAP's ability to inhibit cell death (Liston et al 2001). XAF1 is ubiquitous in normal tissues, but is expressed at low or undetectable levels in many cancer cell lines, suggesting that alterations in the balance of IAP and caspase activities is a common occurrence in the development of the transformed state. Loss of control over apoptotic signaling is now recognized as a critical event in the development of cancer and XAF1 has been suggested to be important in mediating the apoptosis resistance of cancer cells (Liston et al 2001).

Unlike XAF1, DIABLO (also known as Smac) normally resides in mitochondria and is released into the cytosol in response to apoptotic triggers. Following an apoptotic insult, DIABLO/Smac is proteolytically processed into an active form by the removal of its N-terminal signal peptide and is able to promote cytochrome c-mediated apoptosis by binding to and inhibiting XIAP. Unlike XAF1, the functional domains of DIABLO/Smac have been established. The N-terminal tetrapeptide (Ala-Val-Pro-Ile, AVPJ) in DIABLO/Smac shares significant homology with the N-terminal four residues of caspase-9 linker-peptide (ATPF/AVPY) motif, which is also required for its inhibition by XIAP (Srinivasa et al 2001). Indeed, mutation of the very first amino acid abolishes DIABLO/Smac function (Du et al 2000). While BIR3 and BIR2 are associated with caspase-9 and caspase-3 inhibition, respectively (Du et al 2000), the N-terminal four

residues of Smac/DIABLO (AVPJ) are essential for interacting with a surface groove of BIR3 of XIAP and for the activation of the apoptosome (Uren et al 1996; Duckett et al 1996). The binding to the BIR3 domain by two conserved peptides, one from DIABLO/Smac and the other from caspase-9, has opposing effects on caspase activity and apoptosis (Srinivasula et al 2001). Furthermore, the crystal structure of DIABLO/Smac revealed that it forms a homodimer *via* a large hydrophobic interface and can bind the BIR2 domain of XIAP, thus the homodimerization is essential for its function. (Deveraux et al 1999) The caspase-promoting activity of Smac/DIABLO is initially exercised at the level of cytochrome c/Apaf-1/pro-caspase-9 apoptosome, but as a consequence, effects the downstream effector caspases as well (Holcik et al 2001). Over-expression of either DIABLO/Smac or XAF1 does not appear to induce apoptosis, but does sensitize cells to additional cell death triggers (Verhagen et al 2000). It remains to be determined if there exists other cellular pro-apoptotic proteins that share the N-terminal homology with Smac/DIABLO and could modulate IAP activity.

More recently, a serine protease, called HtrA2/Omi, has been reported to be released from the mitochondria and to inhibit XIAP function in a manner similar to that of DIABLO/Smac (Suzuki et al 2001a). Moreover, when over-expressed outside mitochondria, HtrA2/Omi induces atypical cell death, which is neither accompanied by a significant increase in caspase activity nor inhibited by caspase inhibitors, including XIAP (Suzuki et al 2001a). These interesting findings support the contention that, in addition to its role as a caspase activator, HtrA2/Omi is a caspase-independent cell death inducer, the action of which involves serine protease activation (reviewed by Cheng et al 2002).

The activation of Bax via Bid results in the cross-talk between death receptor-triggered and mitochondria-mediated apoptotic pathways (Fig 3). Bax null cancer cells are resistant to TRAIL-induced apoptosis indicating that mitochondrial events are required for apoptosis induced by TRAIL in human cancer cells. Bax deficiency has no effect on TRAIL-induced caspase-8 activation and subsequent cleavage of Bid; however, it results in an incomplete caspase-3 processing because of inhibition by XIAP. Release of Smac/DIABLO from mitochondria through the TRAIL-caspase-8-Bid-Bax cascade is required to remove the inhibitory effect of XIAP and allow apoptosis to proceed. Inhibition of caspase-9 activity has no effect on TRAIL-induced caspase-3 activation and cell death, whereas expression of the active form of Smac/DIABLO in the cytosol is sufficient to reconstitute TRAIL sensitivity in Bax-deficient cell (Deng et al 2002).

XIAP contains three BIR domains and was shown to directly bind and inhibit caspase-3, -7, and -9, but not caspase-1, -6, -8, or -10. This caspase selectivity could be due to the presence of conserved amino acid residues found in the BIR domains and the linker regions that separate them (Sun et al 2000; Sun et al 1999). The structure of the second and third BIR domains of XIAP has been determined by nuclear magnetic resonance. Both structures show a similar hydrophobic zinc-containing core flanked by four or five α -helices respectively, and a number of conserved charged residues. Additionally, the BIRs of *XIAP* contain three anti-parallel β -sheets that are not present in the BIR domain of the closely related HIAP-2. The functional relevance of these differences remains to be determined.

XIAP is known to modulate cell surface receptor-mediated death pathways by inhibiting caspase-3 and -7 (Deveraux, et al 1998) and Bax/cytochrome C mitochondria

pathway by inhibiting caspase 9 (Roy et al 1998; Deveraux et al 1999). *In vitro* kinetic studies have shown that XIAP is the most potent caspase inhibitor in the IAP family, with K_i values of 0.2 to 0.8 nM as compared to a 1 to 20 nM range for the other members of the IAP family (Deveraux and Reed 1999). The caspase-3 and -7 inhibiting activity of XIAP was localized to the second BIR domain (BIR2) but also requires residues found in the linker region that precedes BIR2 (Sun et al 2000; Sun et al 1999). It was shown recently that the isolated BIR3 domain is sufficient to potently inhibit caspase-9 (Sun et al 2000). Furthermore, while investigations into the three-dimensional structure of BIR2 and BIR3 of XIAP indicated that these two domains are very similar, different sets of amino acid residues were found to be critical for the inhibition of distinct caspases. This suggests that BIR domains within XIAP may have distinct functions and that XIAP inhibits caspase-3 and caspase-9 in a different manner. The structural basis of BIR2 and caspase-3 or -7 interaction was recently determined (Chai et al 2001; Huang et al 2001; Riedl et al 2001). This interaction is mediated by residues found in the linker region and not in the BIR2 itself. In fact, the BIR2 domain could be replaced with unrelated protein, such as GST, without effecting the binding to caspases suggesting that the function of BIR2 is perhaps only to stabilize the XIAP/caspase binding.

In addition to the inhibition of caspases, recent discoveries from several laboratories suggest that XIAP is also involved in a number of other biologically significant cellular activities including modulation of receptor-mediated signal transduction and protein ubiquitination (Yang et al 2000; Deveraux and Reed 1999).

As mentioned before, several members of the IAP family are characterized by the presence of a COOH terminal RING Zn finger motif defined by seven cysteines and one

histidine (e.g. C₃HC₄) that can coordinate two zinc atoms (Borden 2000). Interestingly, RING finger proteins have been recognized recently as the largest class of the E3 ubiquitin ligases playing pivotal roles in diverse cellular processes (Joazeiro and Weissman 2000). Orderly protein degradation in the proteasome is an essential cellular process that is required for a number of cellular functions including cell cycle, activation of transcriptional factors, and removal of damaged or misfolded proteins (Hochstrasser 2000). The protein is tagged with polymerized chains of 8 kDa ubiquitin molecules through a series of enzymatic reactions including activation of ubiquitin (by ubiquitin-activating enzyme, E1) and its addition (by ubiquitin-conjugating enzyme, E2) to a Lys residue in the target protein that has been recognized by a ubiquitin protein ligase, E3. The specificity of the ubiquitination process is determined by numerous substrate-specific E3 enzymes, each recognizing a specific substrate.

1.2.4 Survival signaling

1.2.4.1 Phosphatidylinositol 3 kinase (PI3K)/Akt pathway

PI3K/Akt pathway is activated by diverse growth factor receptors (Leevers et al 1999) and important for the suppression of apoptosis in many cell systems (Fig 6). PI3K is a heterodimer composed of a p85-regulatory and a p110-catalytic subunit. The p110 sequence includes a p85-binding region, a ras-binding domain and a catalytic core (Walker et al 1999). In mammalian cells, three isoforms of p85 (p85 α , p85 β , p85 γ) and p110 (p110 α , p110 β , and p110 γ) have been cloned, and found to differ in their activation mechanisms by extracellular agonists, substrate specificity, and subcellular and tissue

distribution. PI3K was first discovered as a lipid kinase that phosphorylates phosphoinositides (PtdIns) at position 3 of the inositol ring to generate the 3-phosphoinositides PtdIns-3-P, PtdIns-3,4-P₂, and PtdIns-3,4,5-P₃ (PIP₃). PIP₃ acts as a second messenger by binding to the pleckstrin homology (PH) domain of Akt (Stokoe et al 1997; Stephens et al 1998). As a consequence, the conformation of Akt is altered, and allowing its threonine 308 and serine 473 residues to be phosphorylated by phosphoinositide-dependent kinase-1 (PDK1) and integrin-linked kinase (ILK), respectively. This signaling process is often referred to as the PI3K/Akt pathway. Numerous studies have shown that Akt is a major target of PI3K. The activated Akt functions to promote cell survival by inhibiting apoptosis via phosphorylation and inactivation of several target proteins including Bad, a pro-apoptotic member of the Bcl-2 family, Glycogen synthase kinase-3 (GSK-3), the forkhead transcription factor (FKHR) and caspase-9 (Asselin et al 2001; Cheng et al 2002).

Akt, also known as protein kinase B (PKB), is a subfamily of serine/threonine protein kinases (Bellacosa et al 1991; Jones et al 1991a & 1991b; Nakatani et al 1999). Three members, Akt/AKT1/PKB α , AKT2/PKB β , and AKT3/PKB γ , have been identified in this family. They share a similar structure with >85% homology, which contains an N-terminal PH domain, a central kinase domain, and a serine/threonine-rich C-terminal region. The PH domain and C-terminal region between these three isoforms are more diverse (homology 73% ~84%) than the kinase domain (homology 90%~95%), suggesting that PH and C-terminal regions may represent functional difference between AKT1/Akt, AKT2 and AKT3 (Cheng et al 1992). Indeed, the three isoforms clearly have been shown to have different biological and physiological function: (a) *AKT1* expression

is relatively uniform in various normal organs whereas high levels of *AKT2* and *AKT3* mRNA are detected in skeletal muscle, heart, placenta and brain (Cheng et al 1992; Nakatani et al 1999; Bellacosa et al 1995; Cheng et al 2002) and (b) Akt2- and Akt1-deficient mice displayed different phenotypes. Akt2^{-/-} mice are born without apparent defects, but develop peripheral insulin resistance and non-suppressible hepatic glucose production, resulting in hyperglycemia and by inadequate compensatory hyperinsulinemia (Cho et al 2001a) similar in some important features to type 2 diabetes in human. The phenotypic characteristics of Akt2 knockout mice are not compensated by the presence of Akt1 and Akt3 (Cho et al 2001b), reflecting differences of substrate specificity in insulin-responsive tissues. In contrast, *Akt1*-deficient mice did not display a diabetic phenotype (Chen et al 2001; Cho et al 2001b). Nevertheless, results indicate that there are non-redundant functions between the three isoforms of Akt in certain tissues and/or cell types.

It has been shown that Akt is activated by a variety of stimuli in a PI3K-dependent manner and is essential for cell survival (Franke et al 1995; Burgering et al 1995; Meier et al 1997; Liu et al 1998). Activation of Akt by growth factors depends on the integrity of the Akt PH domain, which binds to PI3K products PtdIns-3,4-P2 and PIP3, and on the phosphorylation of Thr³⁰⁸ (Thr³⁰⁹ in AKT2 and Thr³⁰⁵ in AKT3) and Ser⁴⁷³ (Ser⁴⁷⁴ in AKT2 and Ser⁴⁷² in AKT3) by PDK1 and ILK (Chan, 1999). The activity of Akt is negatively regulated by *PTEN* (phosphatase and tensin homologue; detected on chromosome 10) that is mutated in about 60% of all solid tumors including human endometroid ovarian carcinoma. *PTEN*, a tumor suppressor gene, encodes a dual-specificity protein and lipid phosphatase that dephosphorylates phosphotyrosine,

phosphothreonine and PIP3. Thus PTEN reduces intracellular levels of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 in cells by converting them to PtdIns-4-P1 and PtdIns-4,5-P2 respectively, thereby inhibiting the PI3K/Akt signaling pathway (Li et al 1998c; Stambolic et al 1998; reviewed by Cheng et al 2002). Loss of expression or mutational inactivation of PTEN leads to the constitutive activation of Akt via enhanced phosphorylation of Thr-308 and Ser-473.

Another phosphatase is SHIP [Src homology 2 (SH2)-containing inositol 5-phosphatase], a 145-kDa protein highly expressed in hematopoietic cells. It specifically hydrolyzes the 5'-phosphate from PtdIns 1,3,4,5-tetraphosphate and PtdIns 3,4,5-trisphosphate, the predominant products of PI 3-kinase. SHIP over-expression has been shown to inhibit Akt activity, whereas SHIP null cells exhibit sustained Akt activity. SHIP has also been shown to down-regulate pro-survival and proliferative signals *in vivo*. The mechanism by which SHIP is regulated remains controversial, although it is known that the molecule must be recruited from the cytoplasm to the plasma membrane for activation (Gardai et al 2002).

Fig 6 The PI3K/Akt pathway activation by growth factors

Cytokines leads to PI3K activation and conversion of PI-4,5-P2 to PI-3,4,5-P3 (PIP3). PIP3 is necessary for recruitment of Akt, ILK, and PDK1 to the plasma membrane. Akt is then phosphorylated by PDK1 and ILK on Thr³⁰⁸ and Ser⁴⁷³, respectively. Activated Akt is involved in modulating a number of cellular pathways, the end results of which is protection of the cell from apoptosis and/or stimulation of cell survival. Phosphatases PTEN and SHIP reduce intracellular levels of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 in cells by converting them to PtdIns-4-P1 and PtdIns-4,5-P2 respectively, and by which inhibit PI3K/Akt pathway.

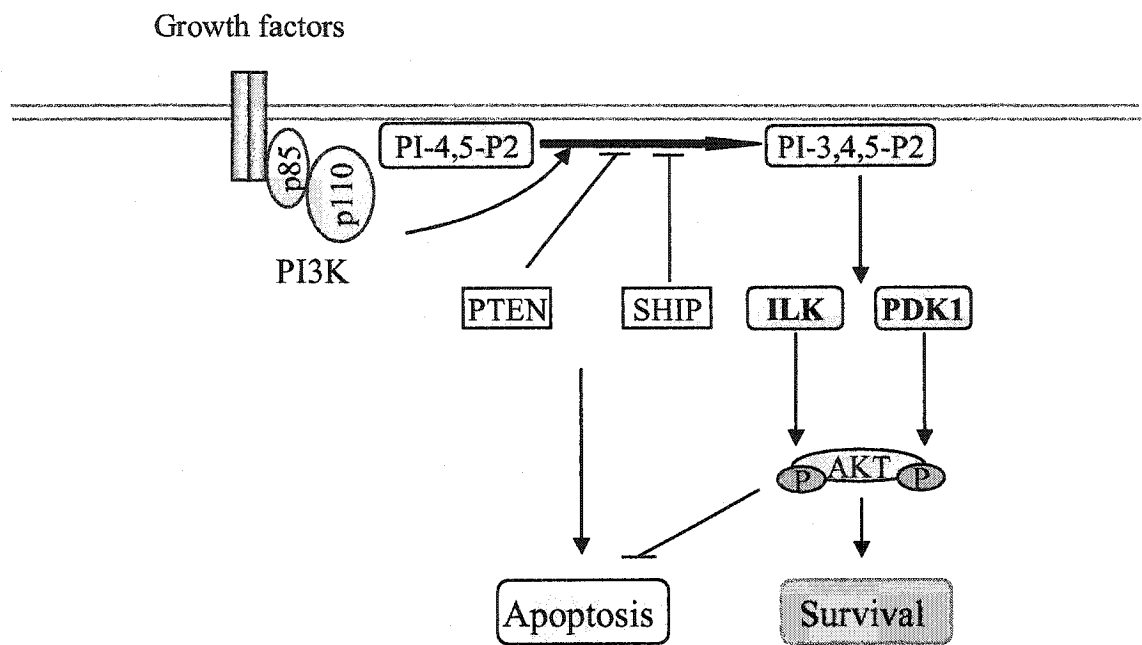


Fig 6

1.2.4.2 Integrins

Integrins (Dedhar 1999) provide a physical link between the extracellular matrix and the cell cytoskeleton. Interaction of cells with the extracellular matrix (ECM) results in the regulation of cell growth, differentiation and migration by coordinated signal transduction through integrins and growth-factor receptors. Integrins achieve signaling by interacting with intracellular effectors that couple integrins and growth factor receptors to downstream components. One well-studied factor is focal adhesion kinase (FAK), but recently another protein kinase, integrin-linked kinase (ILK), has been identified as a receptor-proximal effector of integrin and growth factor signaling. ILK appears to interact with and be influenced by a number of different signaling pathways and this provides new routes for integrin-mediated signaling.

In some cases, specific integrins have selective effects on the efficiency of signal transduction in cell survival pathways (Parise et al 2000). Integrin-mediated cell adhesion is known to regulate gene expression through the activation of transcription factors, a process mediated through ILK. ILK, a down stream effector of the PI3K/Akt, phosphorylates Akt on Ser-473 *in vitro* and *in vivo*, and stimulates Akt activity (Krasilnikov 2000).

1.2.4.3 Peptide and FSH signaling

It is well established that G-protein receptor coupled hormones (such as gonadotropins) increases cellular cAMP level and activates protein kinase A (PKA) in their target cells. A recent report has demonstrated that FSH acts through cAMP,

stimulates phosphorylation and activation of Akt, a PKA-independent signaling pathway in granulosa cells (Richards 2001a). These results provide new and exciting evidence that cAMP acts by PKA-dependent and -independent mechanisms, each of which controls specific kinase cascades. PKA is also known to directly phosphorylate NFκB p65, promote its association with co-activators and consequently increase NFκB transcriptional activity in LPS-challenged 70Z/3 cells (Zhong et al., 1997).

1.3 APOPTOSIS IN THE OVARY

1.3.1 Occurrence of apoptosis in the ovary

Apoptosis was first observed in atretic follicles of the rabbit ovary more than 100 years ago by Flemming, who referred to the process as "chromatolysis" (reviewed by Tilly 1996). Based on ultrastructural evidence obtained by electron microscopy, Hay et al (1976) and O'Shea et al (1978) described the death of ovarian cells in atretic sheep follicles as apoptosis. Zeleznik et al (1989) reported that endonuclease activity was present in rat ovarian nuclei, which was believed to be involved in DNA fragmentation. A role of apoptosis in the ovary did not receive much attention until the appearance of two reports in 1991 describing the occurrence of internucleosomal DNA fragmentation during atresia in rat, porcine and hen ovaries (Hughes and Gorospe 1991; Tilly et al 1991). Since then, apoptosis has been implicated as the cellular mechanism of follicular atresia and luteal regression in every species where these processes occur (Tilly 1996; Hsueh et al 1998; Carambula et al 2002). Morphological and biochemical indices of apoptosis demonstrated that this form of cell death was primarily found in the granulosa,

but not in theca cells of atretic follicles. Theca cell apoptosis was only reported in later stage of atretic follicles (O'Shea et al 1978; McGee et al 1998; Wood and Van Der Kraak 2001). It has become evident that the death of oocytes in prenatal and perinatal ovaries also occurs by apoptosis (Coucouvani et al 1993; Pesce and DeFelici 1994; Morita et al 1999; Eppig 2001). Apoptosis was also found to be the cellular mechanism of luteal cell death during regression of the corpus luteum in cows (Juengal et al 1993) and other species (Tilly 1996; Hsueh 1998; Al Zi'abi et al 2002).

1.3.2 Regulation of apoptosis in the ovary

1.3.2.1 Cell death suppressors

(1) Estrogen, androgen and GnRH

The demonstration that apoptosis occurred in granulosa cells of atretic follicles resulted in vigorous research into the cellular and molecular mechanisms of atresia. Hsueh and colleagues found that controversial atretogenic actions of androgens and GnRH could be substantiated in the study of apoptosis in hypophysectomized rats treated with diethylstilbestrol (DES) implants (Billig et al 1993). DES, a synthetic estrogen, stimulates preantral follicle growth and granulosa cell division in immature rat ovaries. Hsueh found that withdrawal of DES in DES-treated rats caused ovarian apoptosis *in vivo* and this was prevented by estrogen and enhanced by androgen (Billig et al 1993). Similarly, DES withdrawal-induced apoptosis was prevented by FSH and enhanced by GnRH treatment (Billig et al 1994). Ovaries from rats subjected to DES withdrawal contained early antral follicles undergoing atresia, indicating that the progression from

preantral to early antral stages of follicular development can occur in the absence of FSH stimulation, but that the further growth cannot continue without exposure to FSH. The factors capable of stimulating the progression of follicles to the early antral state have not been identified but several growth factors [i.e. epithelial growth factor (EGF), Transforming growth factor alpha (TGF α), Vasoactive intestinal peptide (VIP), Growth differentiation factor-9 (GDF-9); reviewed by (Erickson and Shimasaki 2001) or cytokines (i.e. IL 1- β , IL-6) are capable of inducing some degree of granulosa cell growth and differentiation. Whatever the case, it is clear that in the absence of FSH early antral follicles undergo atresia following DES withdrawal because the granulosa cells die by apoptosis without gonadotropic support.

(2) Identification and expression of XIAP in ovary

XIAP was first identified in ovary by Northern blotting (Liston et al 1996). 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 (Li et al 1998b). 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, in which follicular apoptosis and atresia could occur (Boone et al 1997), 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.

(3) EGF/TGF α , bFGF and NGF

Hsueh and colleagues reported that adult rat antral follicles cultured in the absence of serum undergo apoptosis, which can be prevented, by EGF and basic fibroblast growth factor (bFGF) through a tyrosine kinase-dependent mechanism (Tilly et al 1992b). Peluso subsequently found that EGF induced progesterone production by granulosa cells and suggested that the anti-apoptotic effects of EGF were due to progesterone suppression of intranuclear Ca^{2+} accumulation (Luciano et al 1994). Apoptosis in hen granulosa cells cultured in serum free media was also suppressed by TGF α (Manchanda et al 1996) and this suppression was found to be partially mediated by TGF- α -induced prostaglandin production (Manchanda et al 1996). NGF deprivation increased the expression of caspase-9 and DIABLO/Smac both at the mRNA and protein levels and induced neuronal apoptosis (Troy et al 2001), indicating the caspase-mediating pathway is dependent on the regulated relative expression of components of the pathways including those of caspases, IAPs, and IAP inhibitors, which are regulated by growth factors.

(4) N-Cadherins

Studies by Peluso and colleagues have shown that cell-cell contact between granulosa cells enhanced their survival *in vitro*. This led to the finding that homologous adhesion between N-cadherins was also a survival signal for granulosa cells, independent of progesterone production (Peluso et al 1996). A possible mechanism for N-cadherin suppression of apoptosis has recently been suggested by the observation that both bFGF and N-cadherin suppress apoptosis in granulosa cells via tyrosine phosphorylation of the FGF receptor (Trollice et al 1997). A recent report also demonstrated that PI3K/Akt

pathway is involved in the N-cadherin signaling (Peluso et al 2001) and E-cadherin-mediated cell contact, either directly or indirectly, promotes Akt kinase activity, which in turn, inhibits caspase-3 activation and suppress apoptosis in immortalized granulosa cells.

(5) Vasoactive Intestinal Peptide

Denervation of the ovary leads to disruption of steroidogenesis, ovulation and follicular development, although the precise role of innervation is not known (Lipner 1988). Nerve fibres containing VIP have been identified in developing follicles of several species and VIP receptors are present on granulosa cells (Ahmed et al 1986). VIP stimulates cAMP production, steroidogenesis and plasminogen activator activity in ovarian cells (Trzeciak et al 1986). A potential role for innervation in the survival of follicles was suggested by the fact that VIP suppresses apoptosis in isolated rat and hen follicles (Flaws et al 1995). This effect of VIP was mimicked by cAMP and inhibited by IGF binding protein-3 (IGFBP-3), suggesting that suppression of apoptosis of VIP is a consequence of cAMP-mediated IGF-1 secretion. The expression of both functional subtypes of VIP receptors exists throughout the human female genital tract, with a linear correlation between VIP binding and stimulation of adenylyl cyclase activity (Bajo et al 2000; Reviewed by Igarashi et al 2002).

(6) FSH and intra-ovarian regulators

FSH prevents apoptosis in cultured follicles and is especially important in survival of early antral follicles *in vitro* (Chun et al 1996; Asselin 2000) but other factors independently or in response to FSH stimulation, also have suppressive effects on apoptosis in mature antral follicles. These include IGF-1, EGF, IL-1 β , activin, VIP, GH

and LH/hCG (Chun et al 1996; Hsueh et al 1994; Kaipia and Hsueh 1997; Eisenhauer et al 1995; Chryssikopoulos 2000; Armstrong and Webb 1997). Only FSH completely suppresses the onset of apoptosis in early antral follicles. GH and LH have no suppressive effects on small follicles and IGF-1, activin, VIP, EGF and bFGF can partially but not completely prevent apoptosis in follicles of this size (Chun et al 1996; Kaipia and Hsueh 1997). FSH may act through the stimulation of cAMP to activate PKA or induce transcription of genes controlled by cAMP response elements (CRE). Whether the suppression of granulosa cell apoptosis by FSH is directly mediated by FSH-induced signals or indirectly by the stimulation of local mediators is not yet known and may depend in part on the stage of follicular development. Studies indicated that FSH was an important survival signal in cultured granulosa cell and follicles (Chun et al 1996; Chun et al 1994; reviewed by Markstrom et al 2002). FSH prevented apoptosis in whole cultured follicles and this was abrogated by IGFBP-3, which demonstrated that some component of the survival signal provided by FSH was mediated by IGF-1 (Chun et al 1994). Granulosa cells are the primary source of IGF-1 in the rat ovary (Adashi 1998). Apoptosis in isolated granulosa cells could not be abrogated by IGF-1, but IGF-1 was effective in preventing cell death in whole isolated follicles (Hsueh et al 1994). These findings suggest that theca cells are important in the action of IGF-1 to prevent apoptosis, and that granulosa-theca cell interaction is essential for follicle survival in a manner similar to the "two cell-two gonadotropin" interaction necessary for steroidogenesis. IGF-1 from the granulosa may bind receptors on the theca to stimulate the release of EGF/TGF α that binds to granulosa cell receptors to promote cell survival (reviewed by Markstrom et al 2002).

1.3.2.2 Cell signals of death suppressors

The various agents that can suppress apoptosis in follicles act through different signaling pathways. Gonadotropins and VIP stimulate cAMP production and cAMP suppresses apoptosis. IGF-1, bFGF, N-cadherin and EGF/TGF α act through receptors with tyrosine kinase or PI3K activity that recruit signaling proteins to the activated receptor, leading to cellular effectors such as MAP kinase. The signaling system employed by GH is not entirely known but also appears to operate by recruiting signaling kinases to the activated receptor. Finally, the signaling system of the IL-1 β receptor is reportedly mediated through NF κ B activation (Greene and O'Neill 1999), and leads to the production of nitric oxide and generation of cGMP (Chun et al 1995). These varied signals are all capable of inhibiting apoptosis and may converge on a final effector pathway to have this common effect. The control of granulosa cell survival may not be as complex as this however, since many of these death-suppressing hormones can elicit the local production of IGF-1 or EGF. FSH, LH, VIP, and GH all stimulate the local synthesis of IGF-1. In addition, LH, FSH, bFGF and N-cadherin stimulate the production of EGF/TGF α or its receptor. Finally, IGF-1 stimulates EGF/TGF α production locally in the ovary. Thus, the complex signals may converge at the level of production of one or two local mediators or their receptors. This model does not account for the observation by Chun et al (1996) that the suppressive effects of FSH in early antral follicles cannot be mimicked by IGF-1 or EGF/TGF α alone. However, there are no studies of the combined effects of IGF-1 and EGF/TGF α . FSH may have additional effects not mediated by release of local mediators, including the cAMP-induced stimulation of receptors for those

mediators, or the induction of genes for different components of the signal transduction cascade for EGF/TGF α or IGF-I. FSH may therefore, act to stimulate EGF/TGF α and IGF-I release while simultaneously priming the cell to be more responsive to these factors. If the effects of gonadotropin are mediated by these factors, it remains to be determined what signals elicited by EGF/TGF α and IGF-I are responsible for inhibition of apoptosis in granulosa cells (reviewed by Etgen et al 2001).

1.3.2.3 Direct effects of FSH

(1) FSH and Bcl-2

FSH may have direct anti-apoptotic effects on granulosa cells via the regulation of cell death genes known to regulate apoptosis. Treatment of immature rats with gonadotropin maintained the expression of ovarian Bcl-2 and bcl-X_L mRNA and decreased expression of the bax gene (Tilly et al 1995a), consistent with the rheostatic regulation of these genes towards cell survival (Oltvai and Korsmeyer 1994). Mice with homologous deletions for Bcl-2 and bax have been studied to determine the role of these genes in the ovary. Although ovaries of Bcl-2 knockout mice displayed significantly fewer primordial follicles and oocytes than wild type mice, there was no apparent effect of Bcl-2 ablation on the apoptosis observed in granulosa cells of the adult ovary (Ratts et al 2000; Ratts et al 1995). This suggests that Bcl-2 has a role in the suppression of cell death during fetal development of the ovary when the normal complement of primordial follicles and oocytes is established. The fact that there is a reduction, but not a complete loss, of oocytes and primordial follicles in Bcl-2 knockouts suggests that this gene is not

essential for survival of these cell types. Other genes may be able to compensate for the loss of Bcl-2 in this model. The knockout also demonstrates that the Bcl-2 gene is not required for survival of granulosa cells in the adult ovary, which suggests that other genes such as bcl-X_L may be sufficient for this purpose. Only bcl-X_L mRNA is detectable in granulosa cells of the hen ovary and the levels of bcl-X_L correlate to the susceptibility of hen granulosa cells to undergo apoptosis (Johnson et al 1996). Granulosa cells from immature hen follicles die rapidly in culture and express very little bcl-X_L transcript, whereas cells from mature follicles are resistant to apoptosis and express high levels of the mRNA for this death suppressor gene. This suggests that granulosa cell survival in the hen is mediated by bcl-X_L rather than Bcl-2. Targeted over-expression of Bcl-2 has demonstrated that this death suppressor functions in the mammalian ovary. Mice over-expressing Bcl-2 in their ovaries demonstrated reduced occurrence of apoptotic DNA fragmentation following withdrawal of gonadotropin *in vivo* (Hsu et al 1996). These Bcl-2 over-expressing mice had higher rates of ovulation than wild type mice, and produced larger litter sizes when mated. The incidence of benign ovarian tumors was higher in the Bcl-2 over-expression mice than in wild type mice. These results demonstrated that the loss of follicles by signals to induce atresia was abrogated by Bcl-2, as was the normal loss of follicles to atresia during the induction of follicular development and ovulation (Hsu et al 1996; reviewed by Hsu and Hsueh 2000). Although Bcl-2 knockouts showed that this oncogene is not essential for the prevention of cell death, the targeted over-expression of the gene demonstrates that Bcl-2 does function to prevent apoptosis in ovarian follicles.

(2) FSH and bax

In the mammalian ovary, a high rate of follicular cell apoptosis continues during reproductive life (Hsu et al 2000). Gonadotropins decrease the level of bax mRNA in the ovary (Tilly et al 1995a). Mice deficient in the gene for bax have demonstrated a role for this protein in ovarian cell death. Follicles in bax knockout mice has significant phenotype change and contain degenerating oocytes similar to those found in the later stages of follicular atresia (Knudson et al 1995), but granulosa cells in these antral follicles appear to be resistant to apoptosis. This supports the role of bax as a death-inducing gene in granulosa cells of the ovary. The initiation of apoptosis by DNA damaging signals was shown to absolutely depend on the expression of either Bax or Bak, death promoter members such as myeloid cell leukemia-1 (Mcl-1) as the main ovarian anti-apoptotic Bcl-2 protein, the novel Bcl-2-related ovarian killer (Bok) as the pro-apoptotic protein, as well as Bcl-2-related ovarian death agonist (BOD) and BAD as the proapoptotic ligands (reviewed by Hickman 2002). The activity of the pro-apoptotic ligand BAD is regulated by upstream follicle survival factors through its binding to constitutively expressed scaffold protein 14-3-3 or hormone-induced P11. In contrast, the channel-forming Mcl-1 and Bok-regulated cytochrome c release, together with the recently discovered a novel proapoptotic member of the Bcl-2 protein family Diva/Boo, control downstream Apaf-1 homologs and caspases. Mcl-1 protein was induced after treatment with gonadotropins in granulosa and thecal cells of growing follicles (Leo et al 1999). Elucidation of the role of Bcl-2 members and their interacting proteins in the tissue-specific regulation of apoptosis could facilitate an understanding of normal physiology and allow the development of new therapeutic approaches for pathological states (Hsu et al 2000). Bax or bcl2 are also present in oocytes as well as compounds

(TNF α , Fas) involved in the initiation of apoptosis. However, the molecular and cellular mechanisms triggering oocyte apoptosis are not fully clarified (Driancourt et al 1999; Driancourt and Thuel 1998).

(3) FSH and caspases

Apoptosis, the cellular mechanism of ovarian follicular atresia and luteal regression, is triggered by the activation of caspases. FSH may also directly suppress apoptosis through the regulation of caspases (Asselin et al 2000). While there was no difference between healthy and apoptotic corpora lutea in the distribution or intensity of caspase-3 staining, caspase-3 immunostaining was evident in granulosa cells of atretic but not healthy follicles in a pattern similar to that of the localization of granulosa cell death (Boone and Tsang 1998). These results suggest that the expression of this enzyme is regulated by gonadotropin and may be up-regulated as part of the apoptotic process in granulosa cells. It has also been reported that FSH-induced survival of follicles was associated with decreased expression of the mRNA for caspase-3 and caspase-2, while expression of these caspases was elevated in follicles undergoing apoptosis in serum free culture.

The gene for caspase-1 was expressed at very low levels in the ovary, and levels of this gene did not change in response to apoptotic or survival signals, consistent with recent evidence that caspase-1 is not a principal component of the cell death machinery (Johnson et al 1998). There are many proteolytic substrates for the caspases including PARP, DNA-PK and actin, but to date there are no published studies demonstrating degradation of these proteins during follicular atresia or luteal regression. These proteases appear to be active at some level in all known forms of apoptosis, so the lack of

data concerning proteolytic degradation in the ovary likely reflects the fact that these studies have not yet been done.

(4) FSH and p53

FSH may directly suppress apoptosis through regulation of the p53 gene. Gonadotropin injection to immature rats reduces the level of p53 mRNA expression in granulosa cells, while induction of apoptosis in cultured follicles was associated with elevated p53 mRNA (Tilly et al 1995b). The transcription factor p53 has been known for some time as an anti-oncogenic protein that regulates the DNA synthesis of the cell cycle, and mutations in the p53 gene are commonly found in cancerous cells that have unregulated proliferation (Donehower 2002). Normally, p53 is activated by DNA damage and prevents entry into the cell cycle, allowing time for repair before progression with DNA synthesis. Prolonged activation of p53, however, results in apoptosis in many cell types (Levine 1997). Induction of p53 in immortalized granulosa cells caused apoptosis (Keren-Tal et al 1995; Tajima et al 2002a). It has been demonstrated that p53 can simultaneously induce bax and suppress Bcl-2 gene expression, i.e decreases the the ratio of Bcl-2/Bax, which may therefore alter the balance of cell death genes leading to apoptosis (Miyashita and Reed 1995; Kim et al 2002b). Thus the changes of cellular p53 levels play a critical role in the regulation of apoptotic cell death.

Granulosa cells from equine CG-primed ovaries exhibited marked increases in p53 and Fas protein contents and apoptosis after adenoviral p53-sense complementary DNA infection *in vitro* and were more responsive to Fas activation by an agonistic Fas monoclonal antibody challenge. The control of granulosa cell apoptosis may involve two

consecutive cellular/molecular events: cell cycle arrest at G1/S and exit from G0 into A0 phase, via regulation of the p53 and Fas/FasL death pathways (Kim et al. 1999).

1.3.2.4 Potential cell death inducers

Apoptosis can be induced in follicles at different stages of development by withdrawal of DES or gonadotropin. It is not clear whether such hormone deprivation is sufficient to cause apoptosis or whether this treatment causes the local production of apoptosis inducing factors. Possible inducing factors include androgen (Billig et al 1993) and GnRH (Billig et al 1994), as well as the cytokines IL-6 (Gorospe and Spangelo 1993b), TNF α (Kaipia et al 1996), and FasL (Hakuno et al 1996; Quirk et al 2000). The cytokine IL-6 is produced, and induces DNA fragmentation, in cultured granulosa cells (Gorospe et al 1992; Gorospe and Spangelo 1993). Apoptosis of granulosa cells is the cellular mechanism of ovarian follicular atresia, and cytokines have been implicated as potential atretogenic factors. Previous studies in our laboratory, have investigated the possible role of the cytokine Fas ligand (FasL) and its receptor Fas in apoptosis during ovarian follicular atresia induced by gonadotropin withdrawal (Kim et al 1998). Immunohistochemistry of adjacent histological sections revealed intense positive immunostaining for Fas and FasL in granulosa cells of atretic small and medium antral follicles in a pattern coincidental to the localization of cell death. Intense FasL staining was evident in the theca cells of healthy small antral follicles. An increase in low molecular weight DNA (DNA "ladders") indicative of apoptosis was evident in granulosa cells of the antibody group. Western analysis demonstrated increased levels of both Fas and FasL in the granulosa cells of the antibody group. These results demonstrate that both

Fas and FasL are present in ovarian granulosa cells and that FasL may be the signal that induces granulosa cell apoptosis during atresia at the penultimate stage of ovarian follicular development.

(1) $\text{TNF}\alpha$

$\text{TNF}\alpha$ or ceramide, the putative second messenger of $\text{TNF}\alpha$, induced apoptosis in early antral rat follicles cultured in the presence of FSH (Kaipia et al 1996). Danorubicin, an agent that stimulates *de novo* ceramide synthesis, also induced apoptosis in hen granulosa cells (Witty et al 1996). $\text{TNF}\alpha$, sphingomyelinase, and UV radiation, all of which stimulate ceramide release from sphingolipids, also induced apoptosis (Witty et al 1996). However, in avian granulosa cells ceramide regulates $[\text{Ca}^{2+}]_i$ and progesterone secretion, while the sphingolipid does not appear to play a role in the action of $\text{TNF}\alpha$ (Soboloff et al 1999). These results indicate that ceramide produced by a variety of stimuli was capable of initiating apoptosis, indicating that this second messenger might be a death signal for granulosa cells. Similarly, $\text{TNF}\alpha$, at very high doses, induced apoptosis in cultured mouse luteal cells. This was augmented by prior exposure of the cells to interferon-gamma ($\text{IFN}\gamma$; Jo et al. 1995). Soboloff et al has demonstrated that although $\text{TNF}\alpha$ alone had no effect on cell morphology, it facilitated the reorganization of the granulosa cells into multicellular follicle-like structures in the presence of the growth factor (Soboloff et al 2001). $\text{TNF}\alpha$ and $\text{TGF}\alpha$ interact in the regulation of granulosa cell integrin content and cell survival *in vitro* in a follicular stage-dependent manner. These findings suggest that follicular development is accompanied by a change in the intraovarian role of $\text{TNF}\alpha$; it is atretogenic prior to follicular selection but prevents

follicular demise during preovulatory growth. Previous studies have showed that XIAP is another important intracellular modulator of the TNF α death-signaling pathway in granulosa cells. Its expression is regulated by the TNF α via an NF κ B-mediated mechanism (Xiao et al 2001).

(2) Fas/FasL

The Fas/FasL system was first implicated in ovarian apoptosis by Guo et al (1994) and Quirk et al (1995), who found that monoclonal antibodies to Fas, which mimic the natural ligand, induce DNA fragmentation in cultured human granulosa lutein cells. This effect of the antibody required prior exposure of the cells to IFN γ , presumably to up regulate the Fas receptor (Quirk et al 1995). Fas antibody also induced apoptosis in rat follicles (Hakuno et al 1996). Injection of mice with anti-Fas antibody caused apoptosis in both corpus luteum and follicles, indicating a role for the Fas/FasL system in lutein regression and follicular atresia (Sakamaki et al 1997). Mice bearing the mutation *lpr*, resulting in the absence of functional Fas receptor, were found to have ovaries with excessive numbers of small antral follicles (Guo et al 2002). This may point to a role for Fas in the elimination of follicles at the penultimate stage of development (Sakamaki et al 1997). Kim et al (1998) have also demonstrated that both Fas and FasL are present in ovarian granulosa cells and that FasL may be the signal that induces granulosa cell apoptosis during atresia at the penultimate stage of ovarian follicular development. Granulosa cell Fas/FasL expression was regulated by gonadotropin during ovarian follicular development (Kim et al 1999). Immunohistochemistry localized Fas/FasL in atretic rat follicles and FasL was found in rat oocyte lysate (Hakuno et al 1996) and Fas was increased in cultured granulosa cells exposed to IFN γ . These granulosa cells died by

apoptosis when co-cultured with oocytes, suggesting a potential paracrine regulation of follicular atresia by oocytes. The physiological role of such a mechanism has not been resolved.

1.3.2.5 Signaling by death inducers

A variety of signals may be initiated by apoptogenic factors in the ovary. Androgen presumably acts through its receptor to influence transcription of its target genes. $\text{TNF}\alpha$ and Fas can stimulate ceramide production, leading to the activation of ceramide dependent kinase (Laouar et al 1999; Gamen et al 1998). Both these cytokines can also activate the caspase cascade to induce cell death (Chinnaiyan et al 1996). The IL-6 signaling mechanism is not fully understood, but can induce transcription through IL-6 response elements (Diehl and Rincon 2002). Luteolysis induced by prostaglandin $\text{F}_{2\alpha}$ may be mediated by free radical generation (Carnahan et al 2002). It is difficult to determine which, if any, of these signals is relevant to the induction of apoptosis in ovarian cells. The caspase cascade is likely to mediate the cell death signal of $\text{TNF}\alpha$ and Fas (Gupta, 2003). Ceramide generation can induce cell death, but ceramide is also induced by the survival factor IL-1 β (Santana et al 1996). Similarly, PKC and Ca^{2+} can induce cell death in some systems (reviewed by Minoia and Sciorsci 2001), but these signals are also induced by hormones that stimulate differentiation and steroidogenesis in the ovary. It may be the case that the induction of cell death in the ovary depends on the relative ability of a signal to activate the caspase cascade in the cell. For example, if caspase-3 is not present in a granulosa cell, then ceramide may be a mediator leading to inhibition of steroidogenesis. Following expression of caspase-3, the same signal that

once influenced steroidogenesis might now initiate apoptosis. Whether this is the case requires further study of the components of the caspase system in ovarian cells. Boone and Tsang (1998) showed that mRNA of caspase-3 in the ovary is suppressed by gonadotropin and induced following gonadotropin withdrawal. Caspase-3 has been shown to be localized in luteal cells of healthy corpora lutea and in theca, but not in granulosa cells of healthy follicles. However, immunostaining of caspase-3 was evident in granulosa cells of atretic follicles in a pattern similar to that of the localization of granulosa cell death. There was no difference between healthy and apoptotic corpora lutea in the distribution or intensity of caspase-3 staining. These results demonstrate the presence of caspase-3 in granulosa cells of atretic but not of healthy follicles and suggest that the expression of this enzyme is regulated by gonadotropin and may be up-regulated as part of the apoptotic process in granulosa cells. Caspase-3 has been shown to be functionally required for granulosa cell apoptosis during follicular atresia, but the enzyme is dispensable for germ cell apoptosis in the female (Matikainen et al 2001).

1.3.3 The role of NF κ B and PI3K in the Signaling of FSH and TGF α

As mentioned in the Section 1.2, NF κ B activation regulates the expression of a number of genes involved in the prevention of TNF α -induced apoptosis, such as zinc finger protein A20 (Riachy et al 2002), members of the Bcl-2 family, Bcl-2 homologue Bfl-1/A1 (Lee et al 2002), and XIAP (Xiao et al 2001). FLIP and Bcl-2 are present in both cultured rat granulosa cells and human ovarian surface epithelial cancer cells, and are upregulated by TNF α in a NF κ B-mediated mechanism (Tamatani et al 1999; Wu et al 2002). Overexpression XIAP blocks TNF α -induced granulosa cell apoptosis in the

presence of cycloheximide (Xiao et al 2001). NF κ B activation has been reported in the Sertoli cells in response to FSH (Delfino and Walker 1998). Whether or not NF κ B signaling is involved in FSH-stimulated ovarian follicular development remains to be investigated. In addition, if XIAP expression is indeed regulated by FSH has not as yet been reported.

1.4 HYPOTHESIS AND OBJECTIVES

1.4.1 Objectives

The fate of the developing ovarian follicles (continual growth/ovulation vs. atresia) is determined by the fate of the cells (proliferation vs. apoptosis) within them and regulated by complex actions and interactions of gonadotropins and intra-ovarian factors. Since only a few follicles (only one in human) normally progress to the ovulatory stage in each reproductive cycle, most follicles undergo atresia by granulosa cell apoptosis. While the expression of cell death gene (e.g. Fas/Fas ligand and p53) are important determinants in the granulosa cell apoptosis, studies have shown the presence of various modulators [e.g. Bcl-2 and IAP family] in apoptotic processes. While six IAPs have been identified in mammals, recent studies have shown that gonadotropic stimulation *in vivo* increased XIAP and HIAP-2 expression and follicular growth. Withdrawal of gonadotropin support markedly decreased their expression and induced granulosa cell apoptosis. However, whether these gonadotropin-related changes in XIAP expression during follicular development and atresia are coincidental or critical in determining follicle fate is unclear. Moreover, the signaling mechanism involved in this regulation is

not known. It is also not clear if the gonadotropin effects were a consequence of a direct gonadotropin action on the granulosa cells or of secondary to the secretion and actions of intra-ovarian factors.

The overall objective of the present work was to study the role and gonadotrophic regulation of XIAP expression during follicular development *in vitro*. It was conducted to: (1) define the role of XIAP in the control of granulosa cell apoptosis during follicular growth; (2) determine if TGF α plays a role in the gonadotropic regulation of XIAP expression during follicular development, and (3) determine the signaling mechanism of FSH in the regulation of granulosa cell XIAP expression.

1.4.2 Hypotheses

The expression of XIAP is inducible by FSH during follicular development *in vivo*. XIAP up-regulation in response to FSH suppresses granulosa cell apoptosis and facilitates FSH-induced follicular growth. FSH stimulates granulosa cell proliferation via theca TGF α secretion and action in response to increased granulosa cell estradiol synthesis. FSH-induced XIAP expression is mediated through the NF κ B pathway through activation of PI-3 kinase rather than the classical I κ B kinase.

1.4.3 Scientific innovation and significance

These studies demonstrated, for the first time, the role and hormonal regulation of a novel member of IAP family (i.e. XIAP) in the control of cell proliferation and

apoptosis during follicular development. They also provided important insight into the pathophysiology of female infertility.

1.4.4 Outline of experimental approaches

Two culture systems were used in the present studies, primary granulosa cell (the major cell type of ovarian follicle) culture and individual ovarian follicular culture. By using ovarian follicular culture system, we would be able to exclude the endogenous endocrine factors and examine the influence of gene manipulation of auto- and/or paracrine factors on follicular cell fate and the quality of oocyte. Such a culture system coupled to an adenoviral gene manipulation procedure was established as a useful approach in assessing the role of XIAP in follicular development and atresia. This model has not been applied in other species and the effects of the gene manipulation on the onset and occurrence of apoptosis has not been previously investigated.

To establish the role of XIAP in the regulation of the granulosa cell apoptosis during follicular development and atresia, pre- or early-antral follicles (160-210 μm) were dissected from the immature ovary and cultured in the presence and absence of FSH following adenoviral infection with XIAP full-length sense, or antisense cDNA or LacZ (control). Apoptosis was assessed morphologically (Hoechst nuclear staining), by *in situ* 3'-end terminal dUTP nick end labeling (TUNEL) and on extracted DNA (apoptotic DNA ladder). Follicular cell proliferation was examined by [^3H]-thymidine-incorporation and total cell count. XIAP protein expression was evaluated by Western blot and immunohistochemistry. XIAP mRNA levels were analyzed by RT-PCR. TGF α mRNA levels in follicle sections were assessed by *in-situ* hybridization. SN-50, a cell permeable peptide, NF κ B translocation inhibitor and its inactive control peptide were

used to test the involvement of NF κ B activation in this process. NF κ B-DNA binding activity was assessed by electrophoretic motility shift assay (EMSA). Antibodies to NF κ B subunits (p65 and p50) were used in supershift assay to determine the subunits of NF κ B induced by FSH in granulosa cells. Intracellular NF κ B translocation was assessed by immunocytochemistry and Western blotting of nuclear and cytoplasmic extracts. Neutralizing antibody of TGF α was used to test the involvement of TGF α secretion and action in FSH-stimulated ovarian follicular development.

Chapters 2, 3 and 4 are three published manuscripts in the format of the journals.

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Role and Gonadotrophic Regulation of X-Linked Inhibitor of Apoptosis Protein
Expression During Rat Ovarian Follicular Development In Vitro¹

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ABSTRACT

Although FSH up-regulates follicular cell XIAP expression and suppresses apoptosis *in vivo*, if these events are coincidental or causally related remains to be investigated. The present study examined the role and gonadotrophic regulation of X-linked inhibitor of apoptosis protein (XIAP) expression during follicular development *in vitro*. Follicles (160-210 μm) cultured for 0-6 days with FSH (100 ng/ml) showed significant growth as evident by increases in follicular size, cell number and DNA contents. Follicular XIAP content was low in the absence of FSH but was increased by gonadotropin addition. Apoptosis was evident in follicles cultured without FSH but was suppressed in the presence of the gonadotropin. At low FSH concentration (5 ng/ml), adenoviral XIAP sense cDNA expression increased XIAP and DNA contents, reduced apoptosis, and enhanced the follicular growth. Infection of the FSH-stimulated follicles with XIAP antisense elicited opposite responses. In primary granulosa cell cultures, FSH significantly increased XIAP content, inhibited apoptosis and decreased cell number, a response potentiated by XIAP-sense expression. In conclusion, these studies demonstrated for the first time, that XIAP plays an important role in the regulation of ovarian follicular development. In addition, a follicle culture system coupled to an adenoviral gene manipulation procedure has been established and may prove to be a useful approach in assessing the role of specific genes in follicular development and atresia.

INTRODUCTION

Although a cohort of follicles is recruited to develop during the estrous cycle, only a few are selected to ovulate. The remaining members of the cohort undergo atresia. FSH is an apoptosis suppressor for granulosa cells in vitro [1;2] and in vivo [3] and its presence is critical for the survival of the growing follicles during development [4]. However, the cellular mechanism by which FSH elicits its anti-apoptotic action is still poorly understood.

The inhibitor of apoptosis proteins [IAPs; also termed baculovirus inhibitor of apoptosis repeat (BIR)-containing proteins] is a family of intracellular anti-apoptotic proteins, which were first identified in baculovirus. They are expressed in high abundance in proliferating cells and suppressed in apoptotic ones. To date, six members have been identified in mammals, including X-linked IAP {XIAP, cIAP-3; [5]}, human IAP-1 {HIAP-1, [5], cIAP-2; [6]}, human IAP-2 {HIAP-2, [5], cIAP-1, [6]}, neuronal apoptosis inhibitor protein {NAIP; [7]}, survivin [8] and Livin {[9], termed KIAP in kidney [10]}. The IAPs are characterized by the presence of a caspase recruitment domain (CARD) and an N-terminal BIR motifs, which are necessary for biological activity. With the exception of NAIP and survivin, the IAPs also contain a C-terminal RING-Zinc finger domain believed to be required for protein-protein interactions [11] as well as protein ubiquitination and degradation [12]. Maternal smoking-induced trophoblast apoptosis throughout development is associated with decreased XIAP expression [13]. Increased XIAP expression is believed to play a role in the modulation of Fas ligand-induced apoptosis in malignant glioma cells by a proliferation-inducing ligand (APRIL), a member of the TNF family [14]. Studies on the mechanisms of action

of these anti-apoptotic proteins suggested that IAPs modulate the activities of a group of cysteine proteases known as caspases. XIAP has been shown to be a direct inhibitor of caspase-3 and caspase-7 involved in cell surface receptor-dependent cell death pathway [15;16] and to suppress the mitochondrial (cytochrome c-mediated) pathway by inhibiting caspase-9 activity [17]. Moreover, it has also been shown that mammalian IAPs inhibit caspase-independent apoptosis induced by TNF α in human leukemic cells [6].

Previous studies have demonstrated in the rat ovary that XIAP and HIAP-2 were highly expressed in healthy but not in atretic follicles [18]. While granulosa cell IAP levels were high during gonadotropin-induced follicular development, gonadotropin withdrawal decreased granulosa cell IAP content and induced apoptosis [3]. In addition, gonadotropin-induced, NAIP-mediated suppression of apoptosis in mouse granulosa cells has been suggested to play an important role in the maintenance of oocyte survival during ovarian folliculogenesis [19]. In hen ovarian follicles during follicular development, the highest levels of the inhibitor of T-cell apoptosis (*ita*) gene mRNA within the granulosa cell layer were found in preovulatory (atresia-resistant) follicles, with significantly lower levels detected in pre-hierarchical follicles, a stage at which considerable follicular atresia is observed [20]. The above-described patterns of IAP mRNA expression during follicular development are consistent with a potential role for these genes in protecting granulosa cells from apoptosis and thus maintaining follicle viability. However, the physiological roles of IAPs in follicular development and atresia remain unclear.

In the present studies, we have tested the hypothesis that XIAP expression is essential for the regulation of granulosa cell fate and follicular development by FSH. To

this end, a follicle culture system coupled to an adenoviral gene manipulation procedure has for the first time been established. Using this approach, we have demonstrated that the increase in XIAP expression induced by FSH is critical for the maintenance of normal follicular growth and development.

MATERIALS AND METHODS

Materials

Culture media and reagents were purchased from Gibco Bethesda Research Laboratories (Burlington, ON, Canada). Agarose (low gelling temperature), DNA (calf thymus), Hoechst 33258 compound, Triton X-100, Tween 20, collagenase (Type 1A), DNase 1, RNase A, bovine insulin, human transferrin, ascorbic acid, sodium selenite anhydrous, eCG were obtained from Sigma Chemical Company (St. Louis, MO). The chemiluminescence (ECL) detection kit, [methyl-³H] Thymidine (25 Ci/mmol) and [α -³²P]dd ATP were obtained from Amersham Life Science (Oakville, ON, Canada). Ovine FSH (NIAMDD oFSH-14) was obtained from National Institute of Diabetes & Digestive & Kidney Diseases (Baltimore, MD). Acrylamide (electrophoresis grade), *N*, *N'*-methylene-bis-acrylamide, ammonium persulfate, glycine, SDS-PAGE prestained molecular weight standards (low range), Nitrocellulose membranes, and horseradish peroxidase (HRP) conjugated anti-rabbit and anti-mouse IgG were products from Bio-Rad (Richmond, CA). Rhodamine-conjugated goat anti-rabbit IgG was product of Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mounting medium for fluorescence was purchased from Vector Laboratories, Inc (Vectashield, H-1000; Burlingame, CA). Cell death detection (TUNEL) kit and Terminal End Transferase (TdT) were purchased from Boehringer-Mannheim (Montreal, PQ, Canada) and Alamar Blue dye from Biosource Incorporated (distributor: Medicorp, Montreal, PQ, Canada). The Qiagen Tissue Amp Kit and QIAquick Nucleotide Removal Kit were products of Qiagen (Qiagen Inc., Chatsworth, CA). IM-8 Micro-injector and Joystick (MN-151) as well as

Micromanipulator were purchased from Narishige International Inc (Long Island, NY).

The stage warmer was bought from Linkam Scientific Instruments (Surrey, England).

Adenoviral LacZ, XIAP-sense and anti-sense cDNAs, rabbit polyclonal anti-human XIAP antibodies were generously provided by Dr. Eric LaCasse, Aegera Therapeutics Inc. (Ottawa, ON, Canada). Rabbit polyclonal anti-XIAP antibody was raised against a glutathione S-transferase fusion protein that was expressed in *Escherichia coli* using pGEX vector (Amersham Pharmacia Biotech, Arlington Heights, IL) containing full-length XIAP cDNA. The antibody was affinity-purified by passing through a glutathione S-transferase -XIAP glutathione-Sepharose column. Specificity was confirmed on Western blots (using the antibody-depleted elute from the affinity column), and cross-reactivity with other IAPs was not noted [18]. Construction of recombinant adenovirus was carried out as described previously with some modifications [21]. Briefly, the open reading frame of XIAP was PCR-amplified, cloned in the pCR2.1 vector (Invitrogen, Carlsbad CA), and sequenced. The open reading frame was cut out and ligated into the Sma-1 site of pAdex1CAwt cosmid DNA. The vector was packaged with the Promega cosmid packaging extracts (Promega, Madison, WI) and used to infect *E. coli*. Colonies were picked and screened for the presence of the insert in the antisense orientation relative to the chicken β -actin promoter. CsCl purified cosmid DNA was cotransfected with wild-type adenovirus DNA that was allowed to generate infectious adenovirus DNA only when homologous recombination with cosmid DNA occurred. The final recombinant adenovirus contained a linear, double-stranded genome of 44,820 bp

plus the antisense XIAP insert (~ 1,500 bp). Adenoviral expression system was generated with an Ad E1 insertion vector. Virus titer was determined by the plaque assay.

Follicular isolation and culture

All the animal work was carried out in compliance with the Guide Lines of the Canadian Council on Animal Care. Ovaries from 22-24 day old immature female Sprague-Dawley rats were cut into small pieces and incubated at 37 °C for 30 min in α -minimum essential medium (α -MEM) containing collagenase (Type 1A, 4 mg/ml) and DNase 1 (0.3 mg/ml). The incubation was terminated with the transfer of the ovarian tissues into Leibowitz's L-15 medium with 0.1% (w/v) BSA and follicles (diameter: 160-210 μ m) were dissected out using gauge 28 1/2' needles. To minimize the experimental variation due to damages incurred during the isolation procedures, only round follicles with intact thecal layer on the day of isolation (day-0) and on day-1 of culture were selected for experimentation. Confocal microscopic (Bio-Rad M500; Bio-Rad Laboratories Ltd., Hertfordshire, UK) examination of the selected follicles [following fixation with paraformaldehyde (4 %, v/v, v/v; 30 min, RT) and staining with ethidium bromide (5 mg/ml; 15 min, RT)] revealed that they were at the preantral (75%) and early antral (25%; as evident by the presence of an antral space as large as an area occupied by about 3 granulosa cells) stages of development. Follicles were cultured individually and follicular diameter was measured daily before medium change for 4 to 6 days in 96-well plate in 100 μ l of follicular culture medium [FCM: α MEM medium supplemented with HEPES (10 mM), BSA (0.1%, w/v), rat serum (1 %, v/v), bovine insulin (5 μ g/ml), Transferrin (10 μ g/ml), ascorbic acid (25 μ g/ml), sodium selenite anhydrous (2 ng/ml)

[22] and, non-essential amino acids (1%, v/v), streptomycin-penicillin (0.5%, v/v) and fungizone (0.25%, v/v)] with or without oFSH. Preliminary studies showed that inclusion of ascorbic acid and selenium in the culture medium enhanced follicular integrity, as evident by an increase in the proportion of intact follicles ($65 \pm 5.9\%$ vs. $35 \pm 4.7\%$; $n=3$ experiments; $p < 0.05$) observable at the end of the 6-day culture period. At the end of culture period, follicles were embedded in 2% (w/v) agarose, fixed in buffered formalin phosphate solution (10%, v/v; RT, 3h), stained with Neutral Red (0.1 %, w/v, RT, 3h; to facilitate visualization of follicles during sectioning) and then processed to be embedded in paraffin. Four μm sections of the cultured follicles were also stained with Haematoxylin Phloxine Saffron (HPS) for morphologic examination.

Follicular cell proliferation

1) Follicular cell DNA assay

Cultured Follicles were washed twice with PBS, fixed and sonicated in trichloroacetic acid [5% (w/v), 4°C, 20 min], and finally washed twice with methanol. The DNA pellet, collected by centrifugation (16,000 x g, 10 min), was dissolved in NaOH (0.25 M) and adjusted to neutral pH with HCl (0.25 M). Aliquots of the DNA pellet and calf thymus DNA (standard) were incubated with Hoechst 33258 dye (0.1 $\mu\text{g/ml}$, 5 min in the dark, RT) as described previously [23]. Changes in fluorescence intensity were measured with a Microplate Fluorometer (SPECTRAmax GEMINIXS, Molecular Devices Corporation, Sunnyval, CA) at excitation and emission wavelengths of 356 and 457 nm, respectively. The sensitivity and linearity of the assay was 2 and 4 - 1000 ng/ml, respectively.

2) *Alamar Blue assay*

Alamar Blue is a non-toxic metabolic dye and has been used to monitor changes in cell numbers during tissue or primary cell culture in vitro [24;25]. In the present studies, we have used this assay to monitor the relative increase in cell number during follicular growth under the influence of gonadotropin during a 6-day culture period. The assay was performed as described by Ahmed et al. [25]. To confirm the linear relationship between the changes in Alamar Blue reduction and those of cell number under the experimental conditions of the present studies, Alamar Blue [10% (v/v)] was first tested on primary cultures of rat granulosa cells and human ovarian surface epithelial cancer cell cultures (37°C, 6 h), and subsequently of rat ovarian follicle cultures (37°C, 3 h) maintained under a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO₂. Absorbance at wavelength of 570 nm and 630 nm was measured by a fluorometer (Model: MRX, Dynatech Laboratories Inc. Simi Valley, CA) and their ratio was defined as the cell number equivalence. A linear relationship between ovarian cell number and the extent of Alamar Blue reduction was established with the human epithelial ovarian cancer cell line A2780s ($r^2=0.992$) and isolated rat granulosa cells ($r^2=0.980$). In addition, there was also a direct correlation between Alamar Blue reduction (follicular cell number equivalence) and DNA content ($r^2=0.850$) as well as follicular volume within the range of 2.2 - 25 nl (diameter approximately 160- 360 μm ; $r^2 = 0.933$; Fig. 1). As shown in Fig. 2A, an increase in follicular volume above this range, however, was not associated with a proportional increase in follicular Alamar Blue reduction, an observation consistent with the fact that the increase in follicular volume during early development is mainly a

Fig. 1. Linear relationship between Alamar Blue reduction, follicular volume and DNA content. Follicles ($n = 20$) of different size (from 2.5 to 25 nl in volume) were cultured individually for 24 h. Alamar Blue reagent was added during the last 3 h of culture (1/10, v/v). Follicular volume and DNA content were determined following Alamar Blue reduction assay.

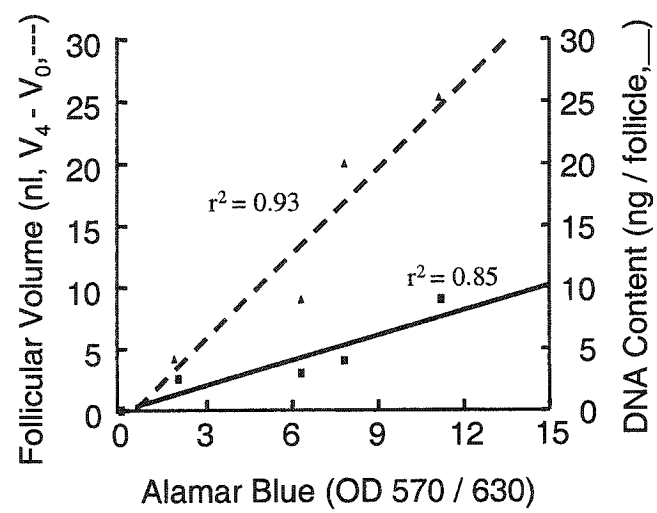


Fig 1

consequence of increased cell proliferation, whereas that at the late stage is due to antral enlargement. No significant change in Alamar Blue reduction was evident throughout the 6-day culture period in the absence of the gonadotropin (Fig. 2A). Maximum daily growth rate was observed on day-3 of culture in the presence of FSH (Fig. 2B).

Follicular adenoviral injection

To assess the role of XIAP in FSH-induced follicular development, XIAP content in the cultured follicles was manipulated by adenoviral XIAP antisense and sense (Myc-tagged) cDNA expression. After a 24h culture in the absence of FSH in a 96 well plate, follicles were transferred onto a cell strainer (100 μ m per mesh, Becton Dickinson Labware, Franklin Lakes, NJ) in a 35mm dish containing FCM. Replication-deficient adenovirus containing LacZ, XIAP sense or antisense full-length cDNAs was injected into the follicles. The volume of the virus injected was less than 10% (v/v) of the calculated follicular volume. Based on the follicular volume and estimated cell number, the amount of virus injected was multiplicity of infection (MOI) of 20 for XIAP antisense cDNA to assure the adequate XIAP down-regulation. The MOI for the virus control (LacZ) was also 20. An MOI of 3 was chosen for XIAP sense cDNA as it was sufficient to up-regulate XIAP expression. FSH (5 ng/ml) was added to the cultures 24 h later and the follicles were cultured for another 3 days. Preliminary studies indicate that follicles cultured with FSH at concentration of 100 ng/ml had higher XIAP level and were more resistant to XIAP down-regulation. In contrast, follicular XIAP level is low in the absence of FSH and less responsive to XIAP up-regulation by adenoviral sense expression. Thus a lower concentration of oFSH (5 ng/ml) was used in subsequent

Fig. 2. FSH increases follicular growth *in vitro*. Rat follicles were cultured for 6 days, with FSH (100 ng/ml) added at the end of day 0 or day 2 of culture. Alamar Blue reagent was added during the last 3 h of culture on each day. Control cultures received no FSH. At the end of the daily culture period, spent medium was collected for spectrophotometer determination (OD 570 nm / OD 630 nm) of cell number equivalence. Changes in DNA content and follicular size were determined as described in Materials and Methods. Follicular volume change on day “n” of culture (A) is defined as the volume difference between day “n” and day “0”, while daily follicular growth of day “n” (B) is defined as that between day “n” and day “n-1”. Following confirmation that no inter-replicate differences exist in each experiment (one-way ANOVA), individual observations from all replicates were pooled for analysis by two-way (repeated measure) ANOVA. Values represent the mean $\pm$ SEM of a total of 43 follicles from three independent experiments. *, $P < 0.05$, **, $p < 0.01$ vs. control (no FSH).

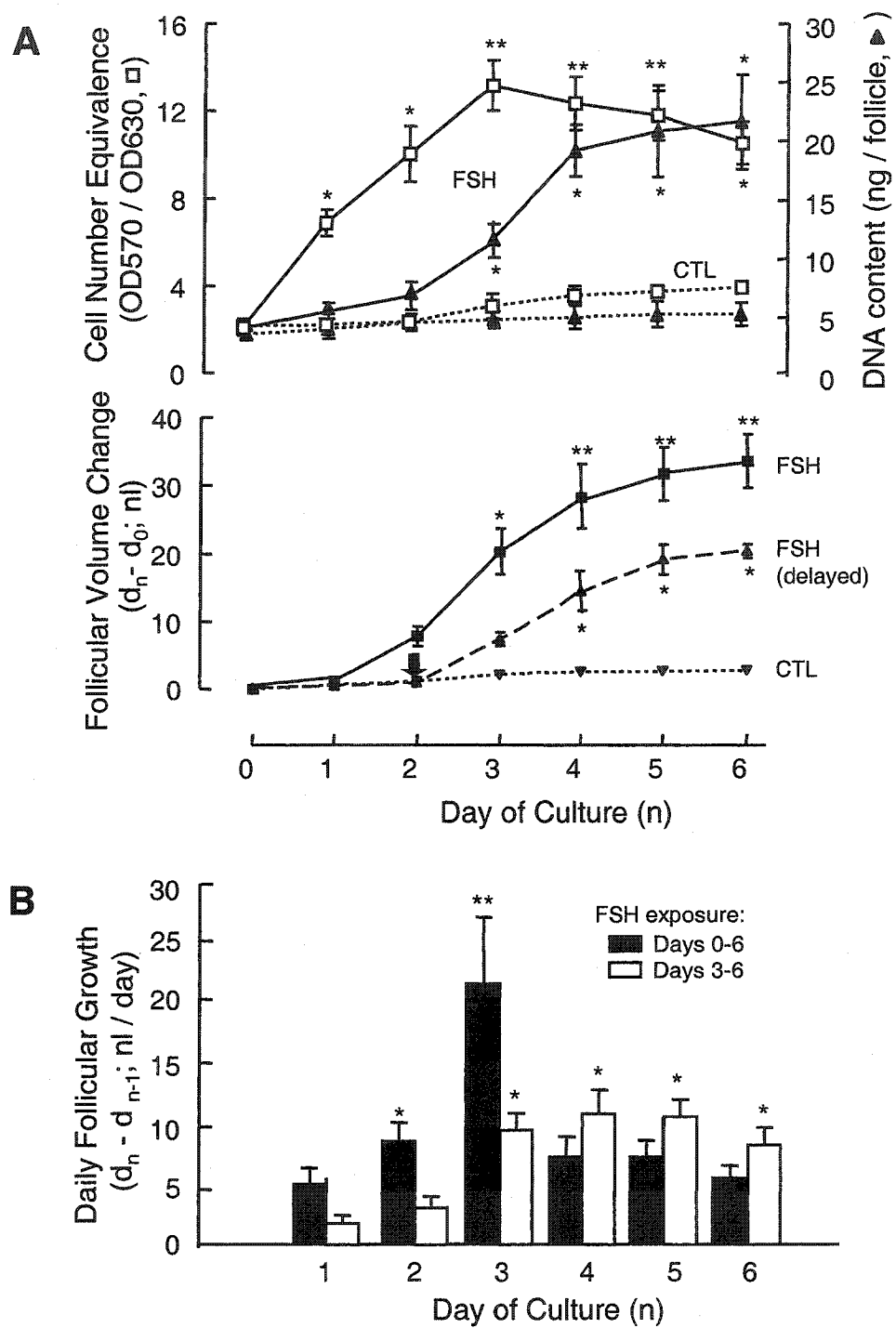


Fig 2

cultures to achieve maximum response to XIAP up- and down-regulation by adenoviral cDNA expression.

To confirm the success of adenoviral infection, follicles injected with the adenoviral XIAP-sense cDNA or LacZ were paraffin sectioned and processed with the same procedure as described in the above section on TUNEL assay up to the incubation step with the blocking reagents. Sections were then incubated (2 h at RT or overnight at 4°C) with HRP-conjugated anti-Myc antibody (1:50; Invitrogen Corporation, Carlsbad, CA) or rabbit anti- β -galactosidase (1:100; ICN Pharmaceuticals Inc., Biochemical Division, Aurora, OH), respectively. Sections of LacZ infected follicles were washed in PBS, incubated with second antibody (HRP-conjugated goat anti-rabbit IgG, 1:200 in PBS; RT, 1 h) and detected by DAB staining [4 min; Boehringer Mannheim Corp., Indianapolis, IN], and counterstained with Methyl Green (100%, v/v; Vector Laboratories Inc., Burlingame, CA). Myc-tag in sections of XIAP-sense injected follicles were directly detected with DAB staining after incubation with primary antibody. In addition, adenoviral LacZ infected follicles (MOI=20; 3 days) were washed twice with PBS, fixed with glutaraldehyde (0.25% (v/v) in PBS; 4°C, 10min), rinsed 4 times with PBS and stained in 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) buffer [PBS (pH 7.2-7.5) containing X-gal (1mg/ml); $K_3Fe(CN)_6$ (5mM); $K_4Fe(CN)_6$ (5mM); $MgCl_2$ (2mM); Triton X-100 (0.05%, v/v); 37°C, 18 h]. Stained follicles were washed twice with PBS and then incubated in a series of glycerol concentrations (10, 20, 40 and 60%, v/v; RT, 4 h for each concentration).

Successful infection of follicular cells with adenoviral LacZ and myc-tagged XIAP sense cDNA was confirmed by X-gal staining (on intact follicles; Fig. 3A & B)

and IHC (on follicular sections), using anti-Galactosidase (87%; Fig. 3C & D) and anti-Myc (82%; Fig. 3E & F) antibodies.

Rat granulosa cell isolation and culture

Immature female Sprague-Dawley rats (24-25 days old) from Charles River Canada (Montreal, PQ) were injected with eCG (15 IU, ip) and ovaries were collected 24 h thereafter in RPMI-1640 medium supplemented with HEPES (10 mM; pH 7.4) and 10% (w/v) fetal bovine serum. Granulosa cells were harvested by follicle puncture as previously described [26], washed and centrifuged ($900 \times g$, 10 min). Viable cells (6×10^5) were plated (6-well plate, Falcon, Becton Dickinson) for 24 h in RPMI 1640 medium with 10% (w/v) FBS under a humidified atmosphere of 95% (v/v) air and 5% CO₂ (v/v) and cultured for various culture duration in serum-free medium with adenoviral LacZ, antisense or sense XIAP cDNA.

Adenoviral infection in primary granulosa cell culture system

Infection of primary granulosa cells with adenoviral XIAP sense and antisense cDNA was performed as described by Kim et al. [26]. Briefly, one million granulosa cells were plated in 60 mm dishes for 24 h and were infected with adenoviral sense or antisense full-length XIAP or LacZ at a MOI of five or twenty, respectively. FSH was added to culture medium 24 h after viral infection and granulosa cells were cultured for another 24 hours. At MOI of 10, the LacZ infection efficiency over 48 h (as determined

Fig. 3. Validation of adenovirus gene delivery in cultured rat ovarian follicles. Adenoviral LacZ infected follicles (MOI=20; 3 days) were washed, fixed, stained in X-gal containing buffer and incubated in series with increasing glycerol concentrations, as described in the Material and Methods section. Panels A and B represent follicles without and with a full cycle of glycerol treatment (10, 20, 40 and 60%), respectively. These results indicate that adenoviral infection was successful. In addition, follicular sections were incubated with rabbit anti- β -galactosidase (Panel C and D) and HRP-conjugated anti-Myc antibody (panel E and F), as described in the Material and Methods section. Panels C and E show images at 40 X, while Panels D and F at 100 X.

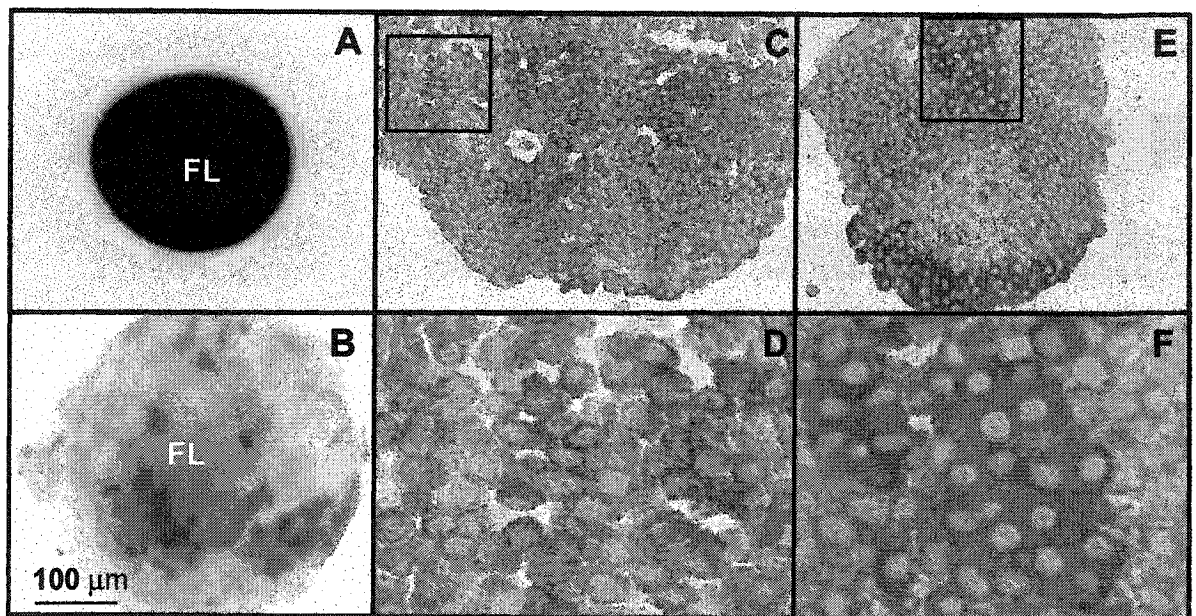


Fig 3

by X-gal assay) was over 90%, and changes in XIAP expression were confirmed by Western blot.

Protein extraction and Western blot analysis

Assessment of XIAP protein contents was performed according to the immunoblotting procedures described by Kim et al. [26] with minor modifications. Briefly, cultured follicles were harvested and lysed mechanically in RIPA buffer [1 x PBS (pH 7.4) containing SDS (0.1%, w/v), sodium deoxycholate (0.5%, w/v), NP-40 (1%, v/v) and protease inhibitors: PMSF (1mM), aprotinin (10 µg/ml) and sodium orthovanadate (1 mM)]. The follicular lysate and harvested granulosa cells were sonicated (10 sec x 3 times on ice) in RIPA lysis buffer. Sonicates were pelleted by centrifugation (14000 x g, 4°C, 30 min) and the supernatant was retained and stored at -20°C. Protein content of the extracts was determined with the Bio-Rad DC Protein Assay Reagent. Samples were mixed with loading buffer, resolved by 10% SDS- PAGE and electro-transferred (30 V, overnight or 80 V, 2 h) onto nitrocellulose membranes, using the Bio-Rad Trans-Blot system. Non-specific binding to the membranes was blocked with blotto [Tris-buffered saline (pH 8.0) with 0.05% (v/v) Tween 20 (TBS-T), 5% (w/v) dehydrated nonfat milk] at room temperature for 1 h. Membranes were then incubated (4°C, overnight) with blotto containing rabbit anti-XIAP antibody (1:2000 dilution), washed in TBS-T (3 x 5 min), incubated in HRP-conjugated secondary antibody (1:5000) in blotto, and washed again in TBS-T twice and then TBS once. Peroxidase activity was visualized with the ECL kit according to manufacturer's instructions. XIAP content was

determined by densitometrically scanning (HP ScanJet 3C; Arlington Heights, IL) the exposed x-ray film (Kodak Canada Inc., Toronto, ON).

Cell Death detection

Hoechst Staining. At the end of granulosa cell culture period, floating cells were collected by aspiration and cells attached to the growth surface were subjected to trypsin treatment [0.05% (w/v) trypsin, 0.53 mM EDTA; 3-5 min; 37 °C). The two cell fractions (floating and attached cells) were combined and an aliquot of this cell mixture was fixed on a microscope slide. At least a total of 200 cells in a randomly selected area in each treatment group were counted. Apoptotic cells were identified based on their typical nuclear morphology. To avoid experimental bias, the “counter” was not aware of the treatment.

Terminal deoxynucleotidyl transferase-mediated dUTP-FITC end labeling (TUNEL).

TUNEL was performed as described previously [27]. Briefly, paraffin-embedded whole ovarian follicle sections (4-5 µm) were mounted on positively charged slides, deparaffinized, hydrated, washed thoroughly for 3 x 5 min in 1 x PBS, and immersed in PBS with 0.3% (v/v) H₂O₂ (RT, 10 min; to inhibit endogenous peroxidase activity). Following three additional 5 min-washes in PBS, the sections were incubated (30 min, RT) in a blocking reagent [Large Volume Dako LSAB Kit; Dako Diagnostics Canada Inc., Mississauga, ON] and immersed in 50 µl of the TUNEL mixture (47.5 µl TUNEL label containing FITC-dUTP and 2.5 µl TUNEL enzyme) in a humidified chamber (37°C, 60 min). They were mounted for fluorescence microscopy with a confocal laser-

scanning system (Bio-Rad 1024). FITC signal in TUNEL positive cells was excited at 488 nm, with images collected within the wavelength range of 506 to 538 nm.

DNA fragmentation analysis

Apoptotic cell death was also assessed on the basis of DNA fragmentation and confirmed through visualization of discrete DNA fragments of 185 base pair multiples on agarose gel electrophoresis. Follicular DNA was extracted using Qiagen Tissue Amp Kit according to manufacturer's instructions. DNA was quantified spectrophotometrically by the absorbance at 260 nm. DNA was end labeled by incubating with TdT and [α - 32 P]ddATP as previously described [13]. Briefly, 500 ng DNA sample was added to a mixture (5 μ l of 5x TdT buffer, 2.5 μ l of 10x CoCl₂, 0.5 μ l TdT enzyme, and 0.5 μ l of 10 mCi/ml [32 P]ddATP and Tris EDTA buffer) to a total volume of 25 μ l, and then incubated at 37°C for 60 min. Unincorporated nucleotides were removed with the Qiagen nucleotide removal kit and the labeled samples were subsequently resolved by 1.8% (w/v) agarose. The gel was dried (3h) and then exposed to a BioRad PhosphorImager, and low molecular weight DNA (<4 kilobase pairs) and genomic DNA were densitometrically quantified. The gel was then exposed to x-ray film at -80°C. To correct for possible uneven gel loading, the ratio of low molecular weight DNA (representing apoptosis) to genomic DNA was calculated for each sample and means of ratios were compared. The intra-observer variability, determined by performing two separate DNA ladder analyses on a same sample, was approximately 5%.

Immunohistochemistry for XIAP

After incubation in TUNEL mixture, sections were immersed in rabbit polyclonal anti human XIAP antibody (1:50) and subsequently in Rhodamine-conjugated goat anti-rabbit IgG (1:200 in PBS; RT, 1 h). XIAP signal (indicated by Rhodamine) was generated with excitation and emission wavelength of 568 and 630 nm, respectively. Confocal Microscopic TUNEL and XIAP images were captured using an imaging software (NIH Image 1.61://rsb.info.nih.gov/nih-image).

Statistical analysis

All experiments were carried out three to four times. Following confirmation that no inter-replicate differences exist in each experiment (one-way ANOVA), individual observations from all replicates were pooled for analysis by two-way (repeated measure) ANOVA (PRISM software version 3.0; GraphPad, San Diego, CA). When XIAP content and the apoptotic DNA fragmentation were expressed as fold of control, it was Arcsine square root-transformed prior to one-way or two-way ANOVA. Granulosa cell number was analyzed by two-way ANOVA. Differences between experimental groups were determined by the Tukey or Bonferroni post-test. The extent of granulosa cell apoptosis between experimental groups was analyzed by Chi-square test.

RESULTS

FSH stimulates ovarian follicular growth in vitro

Follicles cultured for up to 6 days in the absence of FSH exhibited minimal growth (Day 6 vs. Day 0) as evidenced by an absence in the change in follicular size (follicular volume: 3.4 ± 0.55 nl vs. 2.6 ± 0.22 nl, $n = 43$), cell number equivalence (2.8 ± 0.35 vs. 1.5 ± 0.15 , $n = 46$, as determined by Alamar Blue reduction) and DNA content (2.5 ± 0.39 ng/follicle vs. 1.2 ± 0.18 ng/follicle, $n = 40$; Fig. 2A). Addition of FSH (100 ng/ml) to the culture medium significantly increased these parameters (follicular volume: 33.6 ± 3.26 nl, $n = 43$; cell number equivalence: 11.2 ± 2.35 , $n = 46$; DNA content: 21.5 ± 3.39 ng/follicle, $n = 40$; $p < 0.002$ vs. control; Fig. 2A). The increases in cell number equivalence, follicular volume and daily growth rate were maximal on day 3 of culture (Fig. 2A and B). Histological examination of the HPS stained follicles previously cultured over 6 day in the presence of the gonadotropin indicated a well-preserved follicular structure (Fig. 4A-C) containing granulosa cells, theca cells and intact basement membrane (Day 2, Fig. 4B; Day 6, Fig. 4C). Theca cells on day 6 of culture (Fig. 4C) appeared more cuboidal compared to those of follicles of similar stage *in situ* (Fig. 4D) and freshly isolated follicles (Day 0, Fig. 4A). When *in vitro* FSH exposure (100 ng/ml) was delayed for two days (i.e. FSH present on Day 3 to Day 6) the follicles were also responsive to the gonadotropin, although overall follicular growth and the daily growth rate were markedly decreased ($p < 0.002$; Fig. 2A & B).

Fig. 4. Comparison of follicular morphology at day 0 (A), day 2 (B), day 6 (C) of culture in the presence of FSH and ovary in vivo (D). HPS-stained follicular section showing thecal cells (TC), granulosa cells (GC), oocyte (OC) and basement membrane (Δ).

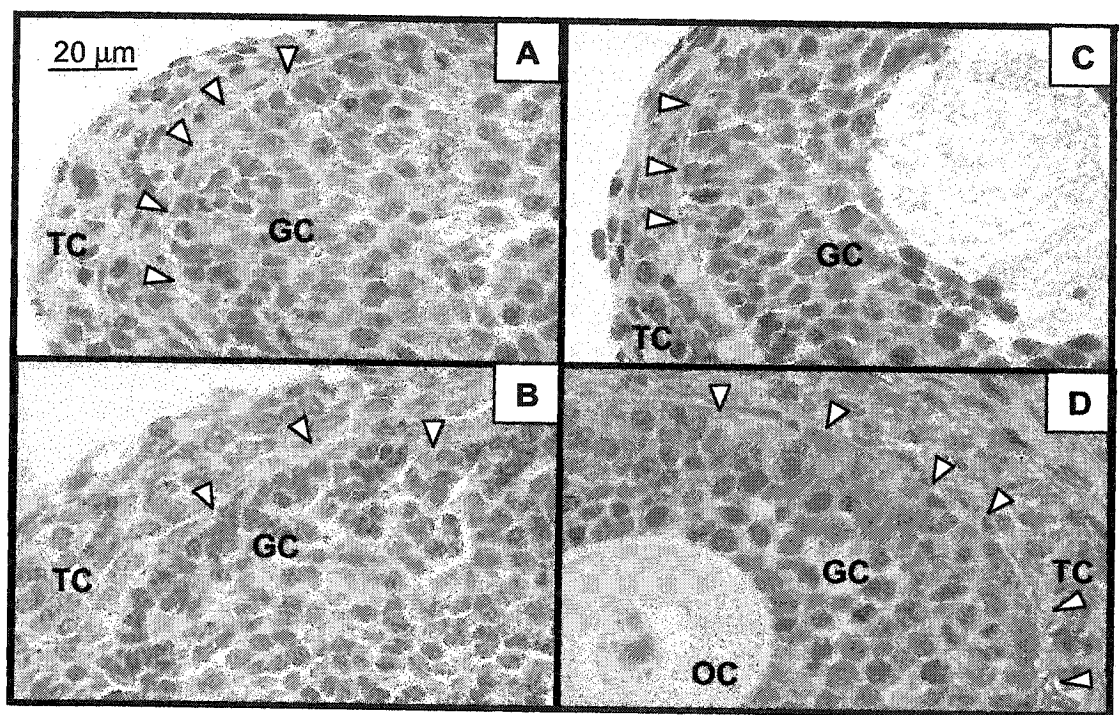


Fig 4

Fig. 5. The effects of FSH (A) and XIAP gene manipulation (B) on rat follicle cultures showing XIAP content and apoptosis. A, follicles were cultured in the absence or presence of FSH (100 ng/ml) for 2 or 4 days. B, follicles were injected with adenoviral LacZ (control, MOI=20) or full length XIAP sense (MOI = 3) or anti-sense (MOI = 20) cDNA and FSH (5 ng/ml) was added to the cultures 24 h thereafter. The follicles were cultured with the gonadotropin for another 3 days, fixed and paraffin sectioned. TUNEL (green) and IHC of XIAP (red) were performed as described in Materials and Methods. Different sizes of the labels “XIAP” and “TUNEL” signify relative intensities of signals. An image from 16 representative follicles is shown for each treatment group.

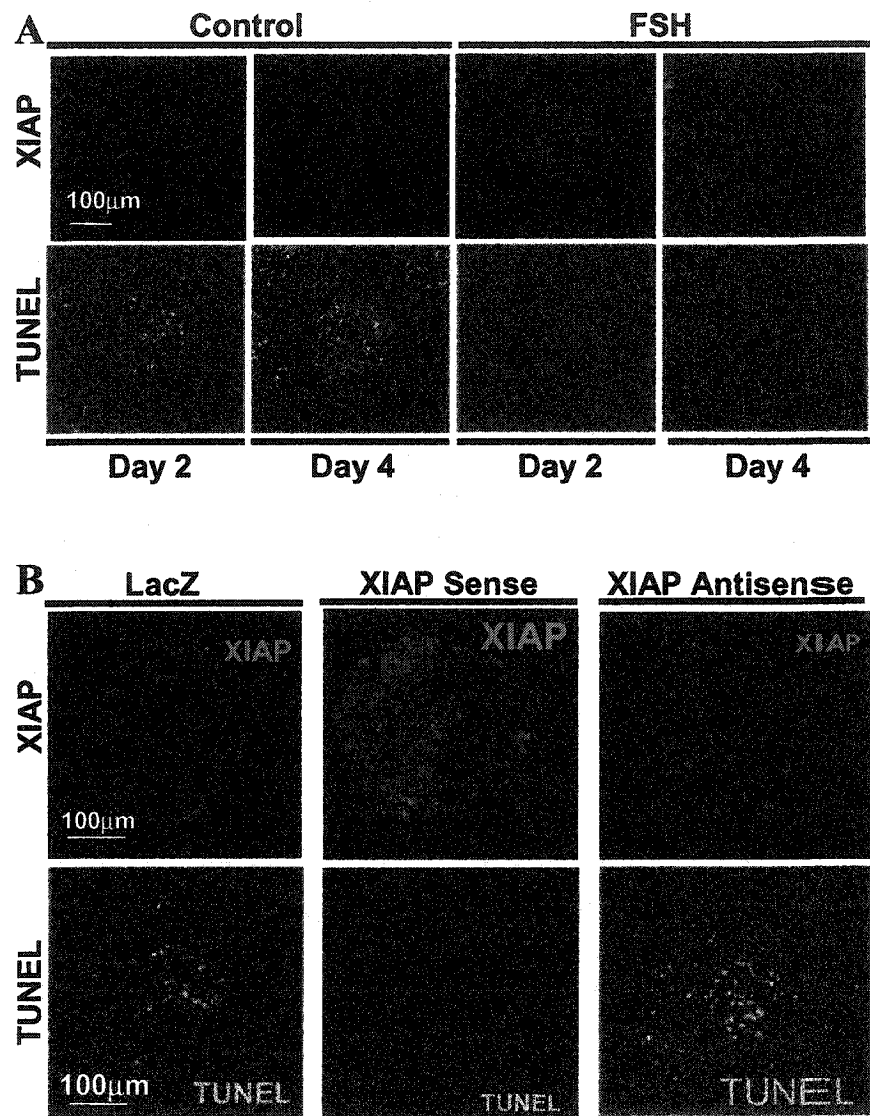


Fig 5

FSH increases XIAP expression in cultured ovarian follicles

To determine if XIAP expression is regulated by the gonadotropin during follicular development and atresia *in vitro*, changes in XIAP content and apoptosis in sections of cultured follicles were examined by immunohistochemistry (IHC) and TUNEL, respectively (Fig. 5A). Whereas follicles cultured in the absence of FSH for 2 and 4 days showed low XIAP immuno-reactivity and detectable apoptotic signal, addition of FSH (100 ng/ml) to the culture medium markedly increased XIAP expression (Fig. 5A) and decreased the apoptotic signal (Fig. 5A). In addition, the gonadotropin also significantly increased follicular XIAP content ($p < 0.05$; Fig. 6) and suppressed apoptotic DNA fragmentation ($p < 0.05$; Fig. 6).

Effects of XIAP down-regulation and over-expression in cultured follicles

Follicles injected with adenoviral XIAP antisense cDNA (MOI = 20) and cultured in the presence of FSH (5 ng/ml) exhibited lower XIAP immunointensity compared to gonadotropin-treated follicles injected with adenoviral LacZ (Fig. 5B). XIAP down-regulated follicles also had a stronger TUNEL-positive signal when compared to the LacZ control (Fig. 5B). XIAP antisense also markedly decreased XIAP contents ($p < 0.05$; Fig. 7) and significantly increased DNA fragmentation ($p < 0.05$; Fig. 7). In addition, infection of the follicles with XIAP anti-sense cDNA significantly attenuated the FSH-induced follicular growth as indicated by a markedly decrease in DNA content ($p < 0.05$) and follicular volume ($p < 0.05$), but was ineffective in the absence of the gonadotropin (Fig. 8). Two-way ANOVA shows a significant FSH effect ($p < 0.001$), antisense effect ($p < 0.001$) and interaction between these factors ($p < 0.001$), brought

Fig. 6. The effects of FSH on rat follicular XIAP content and apoptotic DNA fragmentation. Follicles were cultured in the absence (CTL) or presence of FSH (100 ng/ml) for 4 days. XIAP and tubulin contents were determined by Western blot and apoptosis was determined by 3'-end labeling of DNA fragments,. Representative images (A) and quantitative analysis (B) of follicular XIAP contents (normalized against respective tubulin levels and expressed as fold of control) and DNA fragmentation (expressed by the ratio of < 4K (400 bp) fragmented DNA and respective genomic DNA), are shown. Since XIAP content and the apoptotic DNA fragmentation were expressed as fold of control, it was Arcsine square root-transformed prior to one-way ANOVA. Twenty-five (Western blot) and ten (apoptosis assessment) follicles from same treatment group were pooled and served as one sample. Mean $\pm$ SEM of three independent experiments. *, $P < 0.05$ vs. CTL.

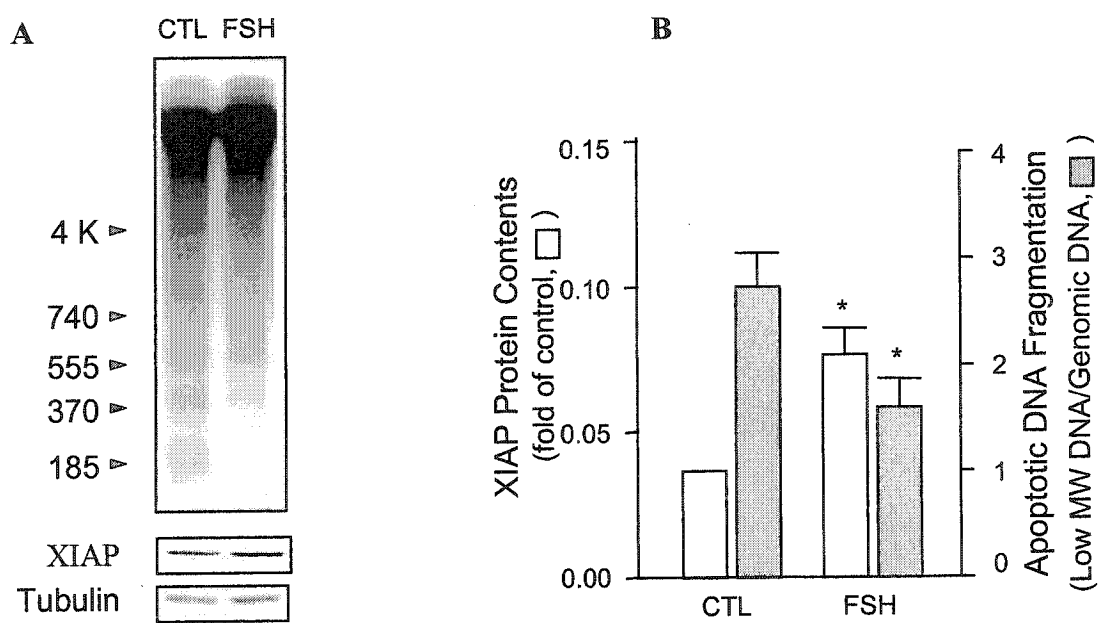


Fig 6

Fig. 7. The effects of XIAP gene manipulation on rat follicular XIAP content and apoptotic DNA fragmentation. Follicles were injected with adenoviral LacZ (LacZ; MOI=20) or full-length XIAP sense (XIAP-S; MOI = 3) or anti-sense (XIAP-AS; MOI = 20) cDNA and FSH (5 ng/ml) was added to the cultures 24 h thereafter. The follicles were cultured with the gonadotropin for another 3 days. XIAP and tubulin contents were determined by Western blot and apoptosis was determined by 3'-end labeling of DNA fragments. Representative images (A) and quantitative analysis (B) of follicular XIAP contents (normalized against respective tubulin levels and expressed as fold of control) and DNA fragmentation (expressed by the ratio of < 4K (4000 bp) fragmented DNA and respective genomic DNA), are shown. Since XIAP content and the apoptotic DNA fragmentation were expressed as fold of control, it was Arcsine square root-transformed prior to one-way ANOVA. Twenty-five (Western blot) and ten (apoptosis assessment) follicles from same treatment group were pooled and served as one sample. Mean $\pm$ SEM of three independent experiments. ⁺, $P < 0.05$; ⁺⁺, $p < 0.01$ vs. LacZ.

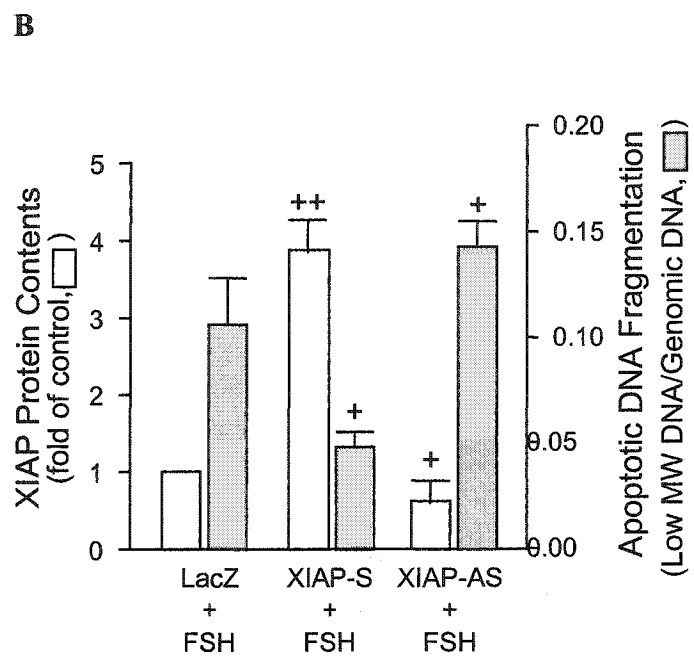
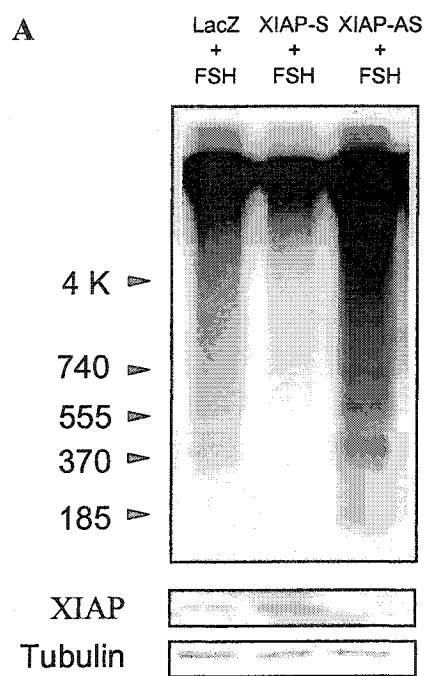


Fig 7

Fig. 8. Effects of XIAP gene manipulation on follicular DNA content (top panels) and volume (lower panels) with/without FSH. After one day of culture in the absence of FSH, follicles were injected with adenoviral LacZ (control, MOI=20) or full length XIAP sense (MOI = 3) or anti-sense (MOI = 20) cDNA and FSH (5 ng/ml) was added to the cultures 24 h thereafter. The follicles were cultured in the absence or presence of gonadotropin (5 ng/ml) for another 3 days. Follicular volume change is defined as the growth between day 0 and day 4 of culture. Following confirmation that no inter-replicate differences exist in each experiment (one-way ANOVA), individual observations from all replicates were pooled for analysis by two-way (repeated measure) ANOVA. Values represent the mean $\pm$ SEM of a total of 36 follicles from three independent experiments. *, $P < 0.05$ (vs. CTL-LacZ); **, $P < 0.01$ (vs. CTL-LacZ); ⁺, $P < 0.05$ (vs. FSH-LacZ).

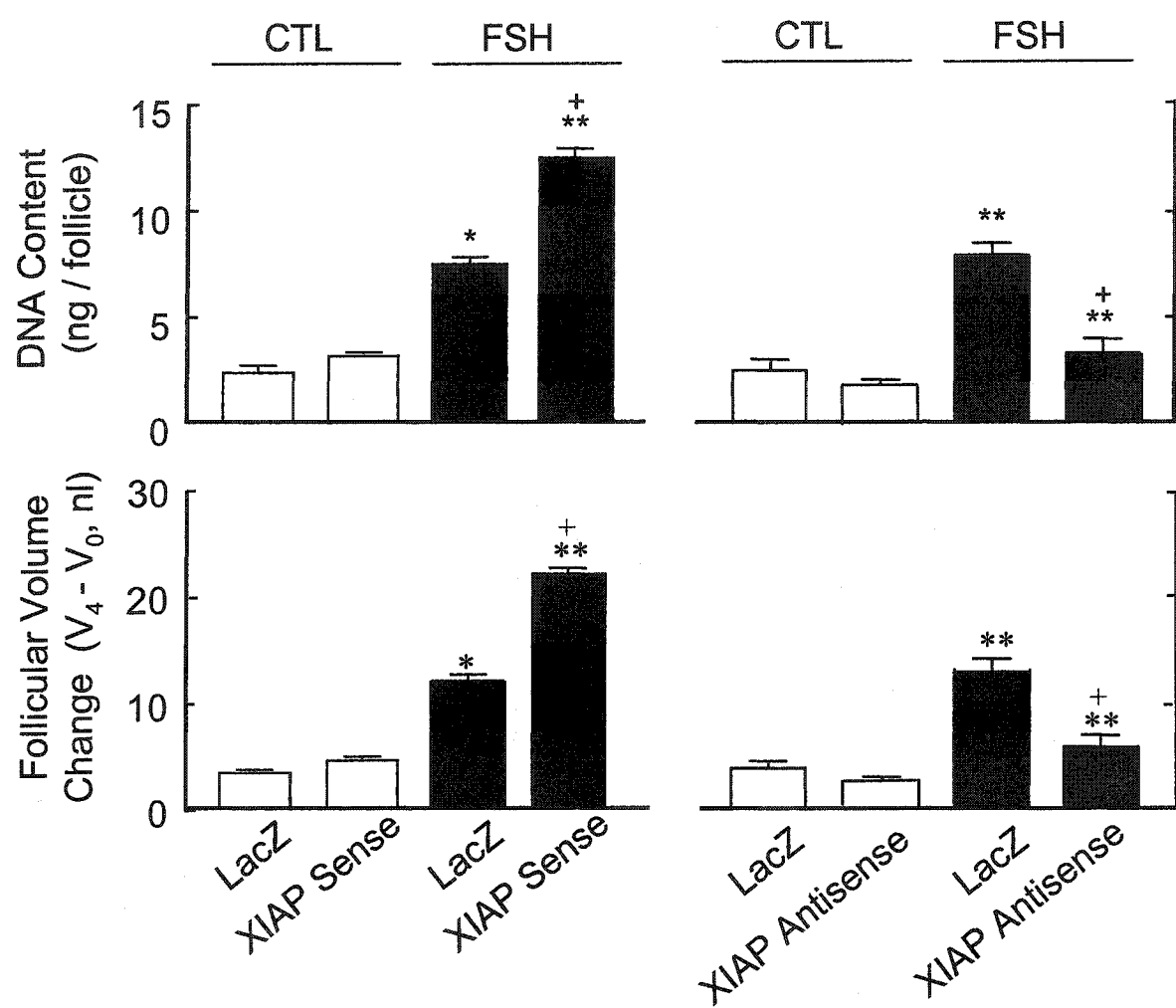


Fig 8

about by the observation that the antisense was more effective in follicles cultured in the presence than in the absence of the gonadotropin.

On the other hand, follicles injected with XIAP sense and subsequently cultured in the low concentration of FSH (5 ng/ml) showed high XIAP immunointensity and less intense TUNEL signal compared to adenoviral LacZ injected follicles (Fig. 5B).

Although a range in the intensity in both XIAP and TUNEL signals was observed within each experimental group, the differences between groups (LacZ vs. XIAP sense vs. XIAP antisense) were obvious. Similarly, XIAP sense infection significantly increased XIAP protein content ($p < 0.01$; Fig. 7) and suppressed apoptotic DNA fragmentation ($p < 0.05$; Fig. 7). The increase in XIAP expression following adenoviral XIAP sense infection was not associated with any significant changes in DNA content and follicular volume ($p > 0.05$; Fig. 8) in the absence of FSH. In contrast, follicles infected with XIAP sense and cultured in the presence of low concentration of FSH (5 ng/ml) showed a marked increase in DNA content (13.28 ± 1.89 ng/follicle vs. 6.85 ± 0.82 ng/follicle, $n = 36$; $p < 0.05$) and follicular volume (22.88 ± 3.29 nl vs. 10.85 ± 2.02 nl, $n = 36$; $p < 0.05$) when compared to the FSH-stimulated and LacZ-infected follicles. Analysis of variance demonstrates a significant FSH effect ($p < 0.001$) and XIAP sense effect ($p < 0.001$), and an interaction between the factors ($p < 0.001$; Fig. 8), due to a greater influence by XIAP over-expression on follicular growth in the presence of the gonadotropin.

Influence of FSH on granulosa cell XIAP expression and apoptosis in vitro

To test if the FSH-induced follicular growth and increase in cell number in vitro is due to a direct action of the gonadotropin on granulosa cells to stimulate proliferation

and/or suppress apoptosis, granulosa cells were cultured for 48-h in the absence or presence of FSH (100 ng/ml). Analysis of variance indicates that although FSH significantly decreased granulosa cell apoptosis when compared to control ($p < 0.01$), total granulosa cell number was markedly lower in the presence of the gonadotropin ($p < 0.02$; Table 1, Two-way ANOVA), irrespective of up- or down-regulation of XIAP ($p < 0.05$; Tukey test).

To determine if FSH has a direct regulatory role in granulosa cell XIAP expression, the influence of the gonadotropin (0~150 ng/ml) on XIAP contents in cultured granulosa cells was assessed during a 24 and 48 h culture period. FSH significantly increased granulosa cell XIAP content in a concentration-dependent manner (Fig. 9). Arcsine square root-transformed two-way ANOVA indicates a significant concentration ($p < 0.01$) but not time ($p > 0.05$) effect. To assess if XIAP may play an important role in the regulation of granulosa cell fate, XIAP level was manipulated by adenoviral expression of XIAP sense and antisense (MOI = 5 and 20, respectively) in the absence and presence of FSH (100 ng/ml). Over-expression of XIAP alone (i.e. in the absence of FSH) markedly suppressed granulosa cell apoptosis ($p < 0.05$; Table 1). In addition, adenoviral XIAP sense cDNA delivery potentiated the anti-apoptotic action of FSH when compared with LacZ infection ($p < 0.05$; Table 1). In the absence of FSH, XIAP antisense expression increased follicular apoptosis when compared to LacZ control ($p < 0.05$; Table 1). Moreover, in the presence of gonadotropin, adenoviral XIAP antisense infection markedly attenuated the cell survival effect of FSH and increased the number of apoptotic granulosa cells ($p < 0.05$).

Table 1. Effects of FSH, LacZ and XIAP cDNA Expression on Cell Number and Apoptosis in Primary Granulosa Cell Culture

Groups	Total cell (x million)		Apoptosis cell (%)	
	Control	FSH (100 ng/ml) *	Control	FSH (100 ng/ml) * *
Medium	1.72 ± 0.20	1.34 ± 0.13	48 (8)	36 (6)
LacZ	1.82 ± 0.22	1.52 ± 0.16	42 (7)	24 (4)
XIAP Sense	1.48 ± 0.14	1.15 ± 0.14 *	24 (4) *	12 (2) *
XIAP Antisense	1.70 ± 0.22	1.25 ± 0.11 *	78 (9.8) *	62 (10.3) *

Mean ± SEM, n = 3 experiments.

Analysis of granulosa cell number by two-way ANOVA indicates a significant overall FSH effect (* p < 0.05). Post hoc Tukey test: * p < 0.05 (XIAP sense or antisense plus FSH vs. XIAP sense or antisense alone).

Chi-square analysis for apoptosis data (% of apoptosis cell in total 600 pooled attached and detached cells) indicates a very significant FSH effect (** p < 0.01).

Chi-square analysis shows difference between treatments:

* p < 0.05 (vs. LacZ in same group, i.e. control or FSH).

Fig. 9. Effects of FSH on granulosa cell XIAP content *in vitro*. Granulosa cells were cultured for 24 and 48 h in the serum-free RPMI-1640 in the presence of different concentrations of FSH (0-150 ng/ml). XIAP and Tubulin contents were determined by Western blot. Representative images and densitometric data of XIAP contents, normalized against respective Tubulin levels and expressed as fold of control, are shown. Since XIAP content was expressed as fold of control, it was Arcsine square root-transformed prior to two-way ANOVA. Mean $\pm$ SEM of three independent experiments. *, $P < 0.05$ vs. control (no FSH).

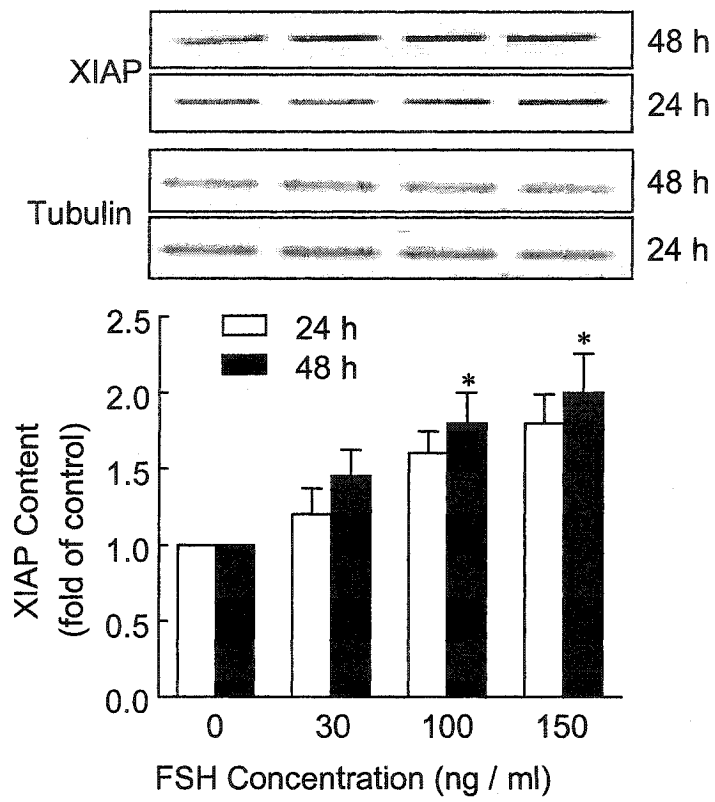


Fig 9

DISCUSSION

FSH is an important survival factor for preantral and antral follicular development *in vivo* [1;28]. Whereas administration of gonadotropin induces ovarian follicular development in immature animals, gonadotropin withdrawal by anti-eCG antibody treatment resulted in not only cessation of follicular growth but atresia [3]. In the present study, we have successfully established a rat follicular culture system and have demonstrated that FSH stimulates rat ovarian follicular growth and induces antral formation in a 6-day culture period. Our findings are consistent with the results of recent studies on cultures of bovine, baboon, mouse, rat and hamster ovarian follicles [29-33]. This culture system may prove to be useful for the assessment of the endocrine control of follicular development and atresia.

The fate of a developing follicle (continual growth & development vs. atresia) is determined by the fate of its cells (proliferation and differentiation vs. apoptosis), which in turn is regulated by their relative expression of “death” and “survival” genes. Thus, follicular development may be a consequence of suppression of cell death genes or over-expression of cell survival genes. It has been demonstrated that follicular atresia is associated with decreased granulosa cell IAP contents, and gonadotropin administration results in the up-regulation of follicular IAPs [18]. Although it is well established that granulosa cell survival and apoptosis are the cellular basis of follicular development and atresia, respectively, whether the changes in the expression of these intracellular anti-apoptotic proteins are coincidental or causally related to these gonadotropin-regulated processes is not known.

Transgenic and knockout animals have been used extensively to assess the role of gene(s) of interest in physiological processes. However, these approaches are often plagued with systemic complications due to overall ill health of the animals, which often affect normal development. IAPs are ubiquitous and found in high abundance in proliferating mammalian cells [34]. Despite the presence of a newly established NAIP knockout mice model [35], no XIAP knockout animal model was available until recently [36] to enable the assessment of the role of XIAP in the gonadotrophic regulation of follicular development and atresia. While there were no apparent differences between the ability of cells from the XIAP-deficient and wild type mice to undergo caspase-dependent or -independent apoptosis, the cellular levels of other IAPs (e.g. cIAP-1 and cIAP-2) were unexpectedly higher, suggesting the existence of a possible compensatory mechanism that leads to the up-regulation of other IAP family members when XIAP expression is lost. However, when and how this compensatory mechanism is triggered is still unclear. By coupling an adenoviral gene delivery system to the above mentioned follicle culture model, we have demonstrated in the present study that XIAP is important in the gonadotrophic regulation of these ovarian developmental processes. In the present study, FSH increased XIAP expression, suppressed apoptosis and stimulated follicular growth (as evident by increases in DNA content, cell number and follicular volume). In contrast, antisense XIAP decreased FSH-induced XIAP, induced apoptosis, and prevented follicular development. These findings suggest that XIAP plays an important role in FSH-stimulated follicular development and serves as an anti-apoptotic factor in rat ovarian follicles. Moreover, adenoviral XIAP sense infection in the cultured follicles increased XIAP contents and attenuated follicular apoptosis in both the absence and

presence of the gonadotropin. Follicles infected with XIAP sense and cultured in the presence of low FSH concentration (5 ng/ml) showed a marked increase follicular development (as evident by increases in DNA content and follicular volume) compared to the FSH-stimulated but LacZ-infected follicles. XIAP sense was ineffective in the absence of the gonadotropin. These findings suggest that while XIAP is necessary for the suppression of the apoptosis, it alone is insufficient to promote follicular growth. They do not exclude the possible involvement of other IAPs in the gonadotrophic regulation of follicular development and atresia. In this context, it has been shown that eCG administration up-regulates granulosa cell HIAP-2 expression in immature rats, suppresses granulosa cell apoptosis and induces follicular growth, while gonadotropin withdrawal suppresses HIAP-2 expression and induces apoptosis and follicular atresia [18]. Moreover, suppression of ovarian NAIP expression with antisense oligonucleotides evoked a decrease in the number of morphologically normal ovulated oocytes, implying an indirect involvement of NAIP in germ cells development via enhancement of granulosa cell survival [19].

The mode of action of XIAP in granulosa cell survival during ovarian follicular development has not been investigated, although we have recently shown that XIAP inhibits caspase-3 in ovarian epithelial cancer cells [37; 38; 39]. It is well established that IAPs modulate cell death pathways by inhibiting caspases [15-17; 37-43] and that the BIR domains are essential for their inhibitory action [40]. Specifically, XIAP possesses two different caspase inhibitory activities, which can be attributed to distinct domains within XIAP. The BIR3 of XIAP is a specific inhibitor of caspase-9, whereas BIR2 plus the linker region of BIR1 and BIR2 is specific for caspase-3 and -7 [40-42]. IAPs

suppress Fas ligand-, TNF α -induced apoptosis by directly inhibiting caspase -3 and -7 activation and activity [15-17] but are ineffective in the activation of caspase-1, -6, or -10 [15]. In addition, XIAP has been shown to modulate the cytochrome c/caspase-9-dependent mitochondrial death pathway. The mammalian HIAP-1, HIAP-2 and XIAP are known to interfere with the function of caspase-9 by binding to inactive procaspase-9, thereby preventing its processing and activation [40;43]. However, whether XIAP functions in this manner in the ovary remains to be determined. Interestingly, recent studies have shown that over-expression of XIAP increases phospho-Akt content in cultured rat granulosa cells [39] and human ovarian cancer cells [44]. This raises the possibility that XIAP may exert its anti-apoptotic action via up-regulation of the PI3K / Akt pathway through a caspase-independent mechanism.

Our present findings indicate that although FSH is capable of increasing follicular cell number (as indicated by increased Alamar Blue reduction and DNA content) and growth in a follicle culture system, it failed to stimulate proliferation when added to granulosa cell primary cultures. While the reason(s) for this apparent discrepancy is not clear, the possibility exist that granulosa cells used in the present studies have been exposed to LH (in the eCG preparation) in vivo prior to isolation and culture, resulting in premature luteinization of cell and consequently exit from the cell cycle. Alternatively, it is also possible that, in addition to being a cell survival factor for granulosa cells via its direct action on XIAP expression, FSH may also stimulate follicular secretion of a mitogen(s), which, in turn, increases granulosa cell proliferation and ovarian follicular growth. In this context, it has been demonstrated that FSH-induced follicular DNA synthesis in the hamster is mediated by follicular secretion and action of EGF [45]. In

addition to gonadotropins, various ovarian factors are believed to play an important role in rescuing follicles from apoptotic demise during follicle development [46]. Previous studies from our laboratory have shown that FSH increases follicular TGF α secretion and that this theca-derived growth factor induces follicular growth in vitro [47]. In this context, recent studies using a co-culture of bovine ovarian granulosa and theca cells have shown that the theca cells are essential in protecting granulosa cells from undergoing apoptosis, a process that is follicular stage-specific. These results suggest that theca cells may secrete a survival factor(s) necessary for maintenance of granulosa cell viability, although the identity of this factor is yet to be defined [48]. It is also possible that the regulation of follicular growth by FSH is mediated by the action of TNF α . Previous studies have shown that gonadotropins increase ovarian TNF α content during follicular development [49] and this cytokine is anti-apoptotic and capable of inducing XIAP expression in rat granulosa cells [50]. The intra-ovarian regulation of follicular development and atresia by gonadotropin remains to be fully explored.

In conclusion, a follicle culture system coupled to an adenoviral gene delivery procedure has been established for the first time. Using this approach, we have demonstrated that XIAP plays an important role in granulosa cell fate determination following FSH challenge, and changes in XIAP expression induced by the gonadotropin are critical for the maintenance of normal follicular growth and development.

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CHAPTER 3 BIOLOGY OF REPRODUCTION 66:1672-1680, 2002

Involvement of Transforming Growth Factor α in the Regulation of Rat Ovarian X-linked Inhibitor of Apoptosis Protein Expression and Follicular Growth by Follicle-Stimulating Hormone¹

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ABSTRACT

The expression of X-linked inhibitor of apoptosis protein (XIAP), a member of a family of intracellular anti-apoptotic proteins, is induced by FSH during follicular development in vivo. Whether the XIAP up-regulation by FSH (100 ng/ml) is a direct action of the gonadotropin and is important in the control of granulosa cell proliferation during follicular growth is unclear. The overall objective of the present study is to examine whether the FSH-induced XIAP expression and granulosa cell proliferation during follicular development is mediated by the secretion and action of intra-ovarian transforming growth factor α (TGF α). In rat follicles cultured for 2 and 4 days, FSH stimulated estradiol production, TGF α secretion, XIAP expression and follicular growth. The theca cells are the primary follicular source of FSH-induced TGF α as indicated by in-situ hybridization. Intra-follicular injection of a neutralizing anti-TGF α antibody (50-200 ng/ml; IgG as control) or addition of estradiol antagonist ICI 182780 (0.5-100 nM) to the culture media suppressed FSH-induced XIAP expression and follicular growth. The effect of ICI 182780 could be partially reversed by high concentrations of estrogen (250 and 500 nM). While TGF α (10-20 ng/ml) significantly increased granulosa cell XIAP content and proliferation in primary granulosa cell cultures, FSH alone was ineffective in eliciting the mitogenic response. Our results support the hypothesis that the FSH stimulates granulosa cell proliferation via theca TGF α 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.

INTRODUCTION

Gonadotropins are important endocrine regulators of follicular selection and survival during development [1]. The differential expression of ovarian factors play a central role in these processes by modulating the action of gonadotropins [2]. Whereas dominant follicles continue to grow and eventually ovulate, the remaining members of the cohort undergo atresia, a process characterized by apoptosis initially of the membrana granulosa and subsequently of the theca layer [3]. In vivo studies on the hormonal regulation of follicular atresia have often been complicated by the presence, in the ovary, of follicles at different stages of development, which exhibit variable responses to endocrine and intra-ovarian regulators [4]. The recent establishment of a rat follicle culture system, in which the regulation of the growth of follicles at a similar stage of development can be assessed, has provided a new and valuable approach for investigating the physiologic and molecular basis of the regulatory processes in the ovary [5]. We have demonstrated that whereas FSH stimulated follicular growth in vitro, it failed to increase granulosa cell proliferation in primary cultures [5]. These results suggest that the mitogenic response of granulosa cells to FSH in follicle cultures was not a consequence of a direct action of the gonadotropin, but rather of the synthesis and secretion of a theca-derived factor(s), which in turn stimulated granulosa cell proliferation.

It has been demonstrated that transforming growth factor α (TGF α) controls 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 vs. atresia) [6]. It has also been shown that epidermal growth factor (EGF), TGF α and FSH increase hamster ovarian follicular DNA synthesis and granulosa cell proliferation [7], which were markedly attenuated by addition of EGF-specific polyclonal antibody into the follicle cultures [8]. These results suggest that FSH-induced follicular DNA synthesis is in part mediated by follicular EGF.

The inhibitor of apoptosis proteins (IAPs) are a family of intracellular anti-apoptotic proteins, which were first identified in baculovirus. They include X-linked IAP (XIAP or cIAP-3), human IAP-1 (HIAP-1 or cIAP-2), human IAP-2 (HIAP-2 or cIAP-1), neuronal apoptosis inhibitory protein (NAIP), Survivin, Livin and Kiap [9-12]. IAPs are characterized by the presence of a caspase recruitment domain (CARD) and an N-terminal baculovirus inhibitor of apoptosis repeat (BIR) motif, the latter of which is necessary for biological activity. With the exception of NAIP and Survivin, the IAPs also contain a C-terminal RING-Zinc finger domain believed to be involved in protein-protein interactions [13] as well as protein ubiquitination and degradation [14]. Only a few reports have to date addressed the mechanisms of action of these anti-apoptotic proteins. XIAP, HIAP-1 and HIAP-2 have been shown to be direct inhibitors of caspase-3 and caspase-7 [15] and also to modulate the Bax/cytochrome C death pathway by inhibiting caspase-9 [16].

We have previously shown that IAP contents in granulosa cells are increased during follicular growth in response to eCG stimulation *in vivo* and *in vitro*, and that eCG withdrawal resulted in decreased IAP expression and increased apoptosis in granulosa cells [17]. However, the mechanism(s) by which FSH induced these changes, is not clear. Moreover, if and how TGF α , a well-established cell survival intermediate, plays a role in the gonadotropic control of the granulosa cells fate (survival vs. apoptosis) during follicular development in the rat has not been investigated. The objective of the present study was to assess the secretion and action of ovarian follicular TGF α , using a defined follicle culture system, and to determine whether this intra-ovarian factor is involved in the gonadotropic regulation of XIAP expression and apoptosis in rat granulosa cell during follicular growth *in vitro*.

MATERIALS AND METHODS

Materials

Culture media, fetal bovine serum (FBS), antibiotics, Trizol, dNTP, Muloney murine leukemia virus reverse transcriptase (MMLV-RT), and restriction endonucleases were purchased from Gibco Bethesda Research Laboratories (Burlington, ON, Canada). Oligo dT and RNase inhibitor were products of Ambion Inc. (Austin, TX). HotStarTaq DNA polymerase and polymerase chain reaction (PCR) purification kit were from Qiagen Inc. (Mississauga, ON, Canada). Agarose (low gelling temperature), DNA (calf thymus), Hoechst 33258, ICI 182780 (ICI), Triton X-100, Tween 20, bovine collagenase (type 1A), bovine DNase 1, bovine insulin, human transferrin, ascorbic acid, sodium selenium, eCG were obtained from Sigma Chemical Company (St. Louis, MO). The chemiluminescence (ECL) detection kit and [methyl-³H] Thymidine (25 Ci/mmol) was obtained from Amersham Life Science (Oakville, ON, Canada). Ovine FSH (NIAMDD oFSH-14) was obtained from National Institute of Diabetes & Digestive & Kidney Disease (Baltimore, MD). Acrylamide (electrophoresis grade), *N, N'*-methylene-bis-acrylamide, ammonium persulfate, glycine, SDS-PAGE prestained molecular weight standards (low range), nitrocellulose membranes, and anti-rabbit and anti-mouse IgG-horseradish peroxidase conjugated were products of Bio-Rad (Richmond, CA). Rhodamine-conjugated goat anti-rabbit IgG, mouse monoclonal anti-TGF α antibody and mouse IgG were products of Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mounting medium for fluorescence (Vectashield, H-1000) was purchased from Vector Laboratories, Inc. (Burlingame, CA). In situ cell-death detection kit and Dig-conjugated mProbe kits were purchased from Boehringer-Mannheim (Montreal, PQ, Canada) and Alamar Blue dyes from Biosource Inc. (Medicorp, Montreal, PQ, Canada). Micro-injector system and stage warmer were purchased from Narishige International Inc (Long Island, NY). ELISA kits for TGF α and 17 β -estradiol (E₂) were purchased from

Oncogene Research Products (QIA61, Damstadt, Germany) and R& D Systems (DE2000, Minneapolis, MN), respectively. The estrogen-antagonist ICI 182780 was a product of Tocris Cookson, Inc. (Ballwin, MO). The pCR II-TOPO cloning kit was from Invitrogen (Carlsbad, CA). Cell strainers were purchased from Becton Dickinson Labware, (Franklin Lakes, NJ).

Follicular isolation and culture

Ovaries from 22- to 24-day-old rats were cut into small pieces and incubated (37 °C, 30 min) in α -minimum essential medium (α -MEM) containing collagenase (type 1A, 4 mg/ml) and DNase 1 (0.3 mg/ml). The incubation was terminated with the transfer of the ovarian tissues into Leibowitz's L-15 medium with BSA (0.1%, w/v), and follicles (160-210 μ m) were dissected out using 28.5-gauge needles. Only follicles judged to be normal (i.e., with oocyte and granulosa cells completely enclosed by the basement membrane and the theca layer [5]) were cultured individually for 4 more days in 96-well plate in 100 μ l of follicular culture medium (FCM: α -MEM supplemented with HEPES [10 mM], BSA [0.1%, w/v], rat serum [1 %, v/v], bovine insulin [5 μ g/ml], Transferrin [10 μ g/ml], ascorbic acid [25 μ g/ml], sodium selenium [1 ng/ml] [18], non-essential amino acids [1%, v/v], streptomycin-penicillin [0.5%, w/v] and fungizone [0.25%, w/v]) with or without oFSH. The follicular size was determined daily before the medium change. At the end of culture period, follicles were embedded in 2% agarose, fixed in buffered formalin phosphate solution [10%; room temperature (RT), 3h], stained with neutral red (0.1%, w/v, 3 h) and then embedded in paraffin. The sections of cultured follicles were stained with Haematoxylin Phloxine Saffron (HPS) for morphologic assessment.

Rat granulosa cell isolation and culture

Immature female Sprague-Dawley rats (24-25 days old) from Charles River Canada (Montreal, PQ, Canada) were injected with eCG (15 IU, ip) and ovaries were collected 24 h thereafter in RPMI-1640 medium supplemented with HEPES (10 mM, pH 7.4) and FBS (10%).

Granulosa cells were harvested by follicle puncture as previously described [19], washed and centrifuged ($900 \times g$, 10 min). Cells were plated for 24 h in RPMI 1640 medium with FBS (10%) under a humidified atmosphere of 95% air and 5% CO₂ and cultured for various duration in the absence and presence of adenoviral LacZ, XIAP antisense cDNA or other treatments (i.e. TGF α , Estrogen, FSH and/or ICI). XIAP antisense cDNA construct is the expression vector pAdex1Cawt containing of a full-length, double-strand cDNA, the expression of which will generate a message in the reverse orientation of XIAP mRNA [20].

Alamar Blue Assay

Alamar Blue is a non-toxic metabolic dye [21], and has been used to monitor changes in cell numbers during tissue growth in vitro [22]. In the present studies, we have used this assay to assess the increase in follicular cell number during growth under the influence of FSH during a 6-day culture period. The assay was performed as described previously [21]. Follicles were incubated with Alamar Blue for 3 h and maintained under a humidified atmosphere (37°C, 95% air and 5% CO₂). Absorbance at wavelength of 570 nm and 630 nm were measured, and the ratio (570 nm : 630 nm) was defined as the cell number equivalence.

Follicular Anti-TGF α Antibody Injection

To examine if FSH-induced follicular growth is mediated through the action of TGF α , follicles were transferred onto a cell strainer (pore size, 100 μ m) in a 35-mm dish containing FCM after a 24-h culture in a 96 well plate. They were injected with anti-TGF α neutralizing antibody (normal mouse IgG for control group at different concentrations (0-200 ng/ml) and cultured in the presence of FSH (100 ng/ml).

Animal Preparation for Ovarian TGF α In Situ Hybridization

Immature (22 days of age) female Sprague-Dawley rats (50-60 g, Charles River Canada, Montreal, PQ) were injected with saline (0.9% NaCl) or eCG (15 IU, ip) and 24 h later, with 100 μ l of either normal rabbit serum (NRS, saline and eCG groups) or anti-eCG antiserum (anti-eCG groups). Animals were sacrificed 24 hrs after NRS or antiserum injection. Ovaries were excised, fixed in 10% formalin and paraffin sectioned for TGF α in situ hybridization assessment.

Adenoviral Infection in Primary Granulosa Cell Culture System

Adenoviral gene delivery was performed as described previously [23]. Briefly, after 24 h of plating in RPMI 1640 medium with 10% FBS, cells were infected with adenoviral XIAP antisense cDNA (XIAP-As) or LacZ at a serial of multiplicity of infection (MOI: 10, 20, 30 and 40). At MOI of 10, the LacZ infection efficiency over 48 h (as determined by 5-bromo-4-chloro-3-indolyl- β -D-galactoside [X-gal] assay) was over 90%, and down regulation of XIAP content by adenoviral XIAP antisense was confirmed by Western blot analysis.

Protein Extraction and Western Blot Analysis

Changes in XIAP content were assessed by Western blot analysis as previously described [17]. Briefly, granulosa cells detached from the growth surface were pelleted and lysed in a ice cold lysis buffer (PBS, NP-40 [1%; v/v], sodium deoxycholate [0.05%; w/v], SDS [0.1%; w/v] containing protease and phosphatase as well as kinase inhibitors, PMSF [10 μ M], aprotinin [50 μ g/ml], sodium orthovanadate [1 mM], sodium pyrophosphate [Nappi, 10 mM], leupeptin and pepstatin [both 5 μ g/ml]. Attached cells were lysed by the addition of lysis buffer to the culture dishes. Cells were sonicated briefly (5 sec/cycle, 3 cycles; 0 $^{\circ}$ C), incubated on ice (30 min), and centrifuged (15,000 x g; 30 min.). Sonicates were pelleted and supernatant was retained and stored at -20 $^{\circ}$ C. Protein content of the extracts was determined with the Bio-Rad DC Protein Assay Reagent. Samples were mixed with loading buffer, resolved by 10% SDS- PAGE and

electrotransferred (30 V, overnight) onto nitrocellulose membranes, using the Bio-Rad Trans-Blot system. Non-specific binding to the membranes was blocked with blotto (Tris-buffered saline [TBS; pH 8.0] with 0.05% [v/v], Tween 20 (TBS-T), 5% [v/v] dehydrated nonfat milk) at room temperature for 1 h. Membranes were then incubated (4°C, overnight) with blotto containing rabbit anti-XIAP antibody (1:2000 dilution), washed in TBS-T (3 washes of 5 min each), incubated in HRP-conjugated secondary antibody (1:5000) in blotto, and washed again in TBS-T twice (5 min) and then TBS once (5 min). Peroxidase activity was visualized with the ECL kit according to manufacturer's instructions. Membranes were reprobed with α -tubulin. Both XIAP and tubulin protein signals were quantified densitometrically. The XIAP protein content was determined by dividing its signal intensity by that of the corresponding tubulin protein content to correct for any loading differences between lanes. XIAP content was then further divided by control value (in the absence of TGF α).

TUNEL and XIAP Double-Staining

TUNEL was performed as described previously [24]. Briefly, paraffin-embedded whole ovarian or follicle sections (4-5 μ m) were mounted on positively charged slides, deparaffinized, hydrated, washed thoroughly for 3 x 5 min in 1 x PBS, and immersed in PBS with H₂O₂ (0.3%, RT, 10 min) to inhibit endogenous peroxidase activity. Following three additional 5-min washing in PBS, the sections were immersed in 50 μ l of the TUNEL mixture (47.5 μ l TUNEL label containing FITC-dUTP and 2.5 μ l TUNEL enzyme) in a humidified chamber (37°C, 60 min). After incubation in TUNEL mixture, sections were immersed in rabbit polyclonal anti human XIAP antibody (1:50 in PBS; RT, 2 h or 4°C overnight) and subsequently in rhodamine-conjugated goat anti-rabbit IgG (1:200 in PBS; RT, 1 h). Sections were mounted with mounting medium and examined using an inverted microscope (Model: Olympus IX 70, 20X objective, Olympus America Inc. Melville, NY) equipped with a confocal laser-scanning system (Bio-Rad

1024). FITC signal in TUNEL positive cells was excited at 488 nm and images were collected at 522 ± 16 nm. XIAP signal (indicated by rhodamine) was generated with excitation and emission wavelength of 568 and 630 nm, respectively. Confocal Microscopic TUNEL and XIAP images were analyzed with imaging software (NIH Image 1.61:<http://rsb.info.nih.gov/nih-image>).

[Methyl-³H] Thymidine Incorporation

Incorporation of [methyl-³H] thymidine into DNA, an index for DNA synthetic capacity of cultured rat granulosa cells was determined as previously described [25]. At the end of adenoviral (LacZ or XIAP-antisense, MOI = 0-40; 24 h) infection with or without TGF α (20 ng/ml, 24h), granulosa cells were cultured for an additional 9 h in the presence of [methyl-³H] thymidine (1 μ Ci). The cells were washed twice with RPMI 1640 with and without unlabeled thymidine (100 μ g/ml), fixed with trichloroacetic acid (5% [w/v], 4°C, 20 min), and finally washed twice with methanol. The DNA pellet was dissolved in NaOH (0.25 M), and adjusted to neutral pH with HCl (0.25 M). A 100- μ l aliquot was stored at -20°C for subsequent DNA assay, and radioactivity in the remaining solution was counted to determine the level of radioactivity incorporated. DNA synthetic capacity index was expressed as cpm/ μ g DNA.

DNA assay

The samples (containing DNA) and calf thymus DNA (standard) were incubated with Hoescht 33258 dye (0.1 μ g/ml, 5 min in the dark, RT) as described previously [25]. Changes in fluorescence intensity were measured with a Microplate Fluorometer (SPECTRAMax GEMINIXS, Molecular Devices Corporation, Sunnyvale, CA) at an excitation and emission wavelengths of 356 and 457 nm, respectively. The sensitivity of the assay was 2 ng/ml, and the linearity was up to 1 μ g/ml.

In situ hybridization

Preparation of cRNA probes. Total ovarian RNA was extracted with Trizol reagent, as per manufacture's instruction, and was used for preparation of first-strand cDNA via MMLV-RT. A negative control was included, using the same reaction mixture but without MMLV-RT (substituted with water) to ensure absence of any contaminating genomic DNA in the RNA template. Expression of the TGF α gene was determined by amplification of a 200-base pair (bp) region (170-369 bp) of the rat TGF α gene sequence. Amplification was carried out using the TGF α antisense downstream sequence 5'-CCCAGAGTGGCAGACACAT-3' and the sense upstream sequence 5'-CTCTGCTAGCGCTGGGTATC-3'. The PCR cycling conditions were chosen as 30 sec at 94°C, 1 min at 55°C, and 1 min at 72°C for 35 cycles, followed by a 10-min extension at 72°C. After amplification, TGF α cDNA was cloned into pCR II-TOPO cloning vector and transformed into competent *E.coli* DH5 α cells. The insert was digested with *EcoRI* to verify the presence of the insert. The cDNA was sequenced in both directions to confirm TGF α sequence (Gibco). DIG-labeled ribo-probes for in situ hybridization were synthesized by in vitro transcription, using a DIG RNA labeling kit. Sense TGF α probe was generated by linearizing DNA templates with *HindIII* and in vitro transcription with T7 polymerase. Antisense TGF α probe was generated by linearizing the DNA templates with *Nsi I* and in vitro transcription with Sp6 polymerase. Digestions were monitored on agarose gel (2% [w/v]) and fragments purified using PCR purification columns.

Hybridization analysis. Ovarian tissue and follicle sections were deparaffinized and rehydrated through a graded series of ethanol. Tissue sections were washed with diethyl pyrocarbonate (DEPC)-treated PBS (2 washes of 5 min each), DEPC-treated PBS with glycine (100 mM, 2 washes of 15 min each), DEPC-treated PBS with Triton X-100

(0.3% [v/v], 15 min), and DEPC-PBS (2 washes of 5 min each). Sections were permeabilized with RNase-free proteinase K (20 µg/ml) in TE buffer (100 mM Tris, 50 mM EDTA; pH 8; 30 min), refixed in paraformaldehyde-PBS (4% [w/v], 4°C, 5 min), rinsed twice in PBS, dehydrated, dried at room temperature, and then washed with 4 x SSC (1 x SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.2) containing formamide (50%, 10 min). Sections were hybridized with DIG-labeled antisense or sense (negative control) TGF α cRNA probes as described above. Hybridization buffer (40% [v/v] de-ionized formamide, 4 x SSC, 1 x Denhardt's, 10% dextran sulfate, 1.0 mg/ml yeast tRNA, 1.0 mg/ml denatured salmon sperm DNA and 10 mM DTT) containing DIG-labeled cRNA probe (10 ng/slide) was applied to each slide for hybridization (overnight, 42°C) in a humidified chamber. Slides were then washed in 2 x SSC (3 washes of 15 min each) and 1 x SSC (2 washes of 15 min each) at 37°C. Sections were then incubated (37°C, 30 min) with DNase-free RNase (20 µg/ml) in TEN (10 mM Tris, 1 mM EDTA, 500 mM NaCl; pH 8.0) buffer to remove non-specific binding and washed as follows: 0.1 x SSC (2 x 30 min, 37°C) and TN buffer (100 mM Tris-HCl [pH 7.5] and 150 mM NaCl, 2 washes of 10 min each). Sections were blocked with TN buffer containing 0.1 % Triton X-100 and 2% normal sheep serum for 30 min and incubated (2h, 37°C) in TE buffer containing Triton X-100 (0.1% [v/v]), normal sheep serum (1% [v/v]) and sheep anti-DIG-alkaline phosphatase (1:100 dilution). Sections were again washed with TN buffer (2 x 10 min) and with TNM buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 10 min), covered with 200 µl of color solution [10 ml TNM buffer containing 45 µl nitroblue tetrazolium (75 mg/ml in 70% [v/v] dimethylformamide), 35 µl 5-bromo-4-chloro-3-indolyl-phosphate (50 mg in 100% dimethylformamide), and 1

mM levamisole] and incubated in the dark for 24h. Color reaction was stopped by incubating slides by 10 mM Tris-HCl, 1 mM EDTA (pH 8.0). Six ovaries from three rats in each experimental group (Control, FSH, eCG or eCG plus anti-eCG antibody) were embedded into one block, and three sections from each block were tested. Six follicles per treatment group in each experiment were embedded into one block. Experiment was repeated twice. An image from 18 representative ovaries or follicles is shown for each treatment group.

Enzyme-Linked Immunosorbent Assays

Transforming growth factor α . TGF α ELISA assay was performed according to the manufacturer's instruction. This assay kit has been validated for detection of TGF α in spent medium [26]. Briefly, biotinylated TGF α reporter antibody was added into wells pre-coated with a capture antibody following the addition of the samples or standards. TGF α presented in the sample binds to both capture antibody and the reporter antibody in solution. Wells were washed twice with the wash buffer and once with the rinse buffer, and then incubated (30 min, RT) with streptavidin-horseradish peroxidase, which conjugates to the reporter antibody. Substrate reagents were then added into wells and incubated (30 min, RT) in the dark. Reaction was stopped by the addition of Stop Solution and absorbance at 490 nm was measured with a microplate reader. Samples were accumulated and analyzed together to avoid inter-assay variation. The intra-assay variation was 4.4%.

17 β -Estradiol. The principle of E₂ ELISA was based on the competition between E₂ present in samples and alkaline phosphatase-labeled E₂ for sites on a rabbit polyclonal antibody. The assay was performed according to the manufacturer's instruction. Based on the manufacturer's information and previous report [27], the E₂ ELISA assay is validated to detect E₂ in cell culture medium with an average recovery rate of 98%. Briefly, following incubation of alkaline phosphatase-labeled E₂ antibody with samples or standards (2 h, RT), wells were washed with

Wash Buffer and subsequently incubated with substrate reagents (45 min, RT). The reaction was stopped by the addition of the Stop Reagents and the optical density at 405 nm was assessed by a microplate reader. The intra-assay variation was 5.2%.

Statistical analysis

All experiments were repeated at least three times. Data were subjected to one- or two-way ANOVA (PRISM software version 3.0; Graph Pad, San Diego, CA). Differences between experimental groups were determined by Tukey test. The data on XIAP content, as determined by Western blot analysis and expressed in folds of the control (Fig. 5A), was arcsine square root-transformed prior to two-way ANOVA.

RESULTS

Role of the TGF α on FSH Stimulated Follicular Growth In Vitro

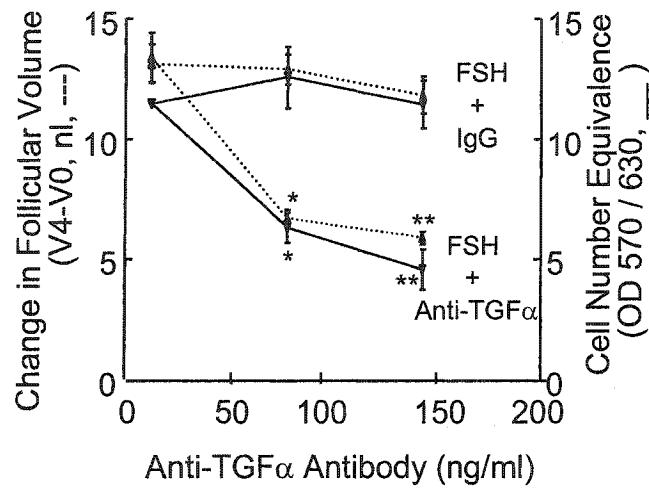
Follicles cultured in a 4-day period in the absence of FSH had minimum growth (Day-4 vs. Day-0: follicular volume: 3.6 ± 0.65 vs. 2.2 ± 0.25 nl, $n = 28$; cell number equivalence: 2.5 ± 0.35 vs. 1.2 ± 0.15 nl, $n = 28$), while the addition of the FSH markedly increased follicular volume (16.5 ± 1.9 nl, $n=30$; $P < 0.001$ vs. control) and cell number equivalence (14.7 ± 1.6 , $P < 0.001$, vs. control). Intra-follicular injection of the anti-TGF α antibody (0-200 ng/ml) markedly attenuated the increases in follicular volume ($P < 0.01$) and cell number equivalence ($P < 0.01$) induced by FSH in a concentration-dependent manner, whereas injection of normal mouse IgG was ineffective ($n=30$ for each treatment group, $P > 0.05$; Fig. 1A). At 150 ng/ml, anti-TGF α antibody significantly suppressed FSH-induced increase in follicular volume (from 12.5 ± 0.9 to 6.1 ± 0.5 nl, $n = 30$, $P < 0.01$) and cell number equivalence (from 11.9 ± 0.7 to 4.8 ± 0.5 nl, $n = 30$, $P < 0.01$) during the 4-day culture period. TGF α (10 ng/ml) alone exerted a small but concentration-dependent increase in follicular growth in vitro ($P < 0.05$; Fig. 1B) and significantly enhanced the response induced by the gonadotropin (10 ng/ml; $P < 0.05$; Fig. 1B).

FSH Stimulates Estrogen-Dependent Follicular TGF α Secretion In Vitro

To further examine if FSH-induced follicular growth is mediated through the secretion and action of TGF α , follicles were cultured in the absence or presence of FSH (100 ng/ml). The levels of TGF α in the spent media on Days 2 and 4 of culture were measured. TGF α levels in follicle cultures with gonadotropin were significant higher than those of the control groups, irrespective of the duration of the culture ($P < 0.01$; Fig. 2A). To determine the cellular source of follicular TGF α , TGF α mRNA levels in ovarian and follicular sections following gonadotropin stimulation in vivo and in vitro, respectively, were examined by in situ hybridization (Fig. 3A).

Figure 1. Role of TGF α in FSH-induced ovarian follicular growth in vitro. A: Anti-TGF α antibody blocks follicular growth. Follicles were cultured for 4 days in the presence of FSH alone (100 ng/ml) or FSH plus different concentrations of anti-TGF α antibody or IgG (as control). Follicular diameter and cell number equivalence were measured at day 0 and day 4. *, $p < 0.05$; **, $p < 0.01$ compared to FSH + IgG at the respective concentration. B: TGF α alone stimulates follicular growth. Follicles were cultured with different concentrations of TGF α $\pm$ FSH for 4 days and follicle diameter was measured at day 0 and day 4. Changes in follicular volume is defined as follicular volume at day 4 less that of day 0 ($V_4 - V_0$). *, $p < 0.05$, **, $p < 0.01$, vs. control (no TGF α) and +: $p < 0.05$ vs. FSH alone (10 ng/ml). Values indicated in A and B represent the Mean $\pm$ SEM of a total of 30 follicles from three independent experiments.

A



B

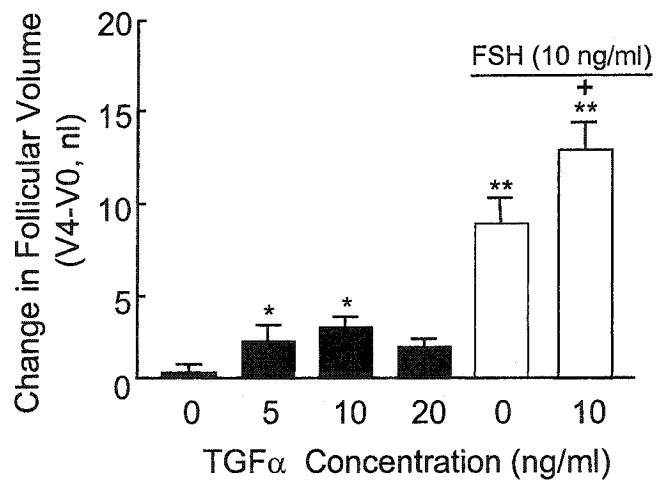


Fig 1

Figure 2. Influence of FSH on follicular estradiol and TGF α secretion in the absence and presence of ICI 182780 (ICI) in vitro. A: FSH increases estradiol and TGF α secretion in cultured follicles. Follicles were cultured with or without FSH (100 ng/ml) for 4 days, and spent media were assayed for TGF α and 17 β -Estradiol by ELISA. B: FSH-induced TGF α secretion was suppressed by estradiol antagonist ICI. Follicles were cultured individually with or without FSH and/or ICI (0.1 μ M) for four days. Spent media were assayed for TGF α , as above. Results are Mean $\pm$ SEM from three independent experiments. *: $p < 0.05$; **: $p < 0.01$ compared to CTL.

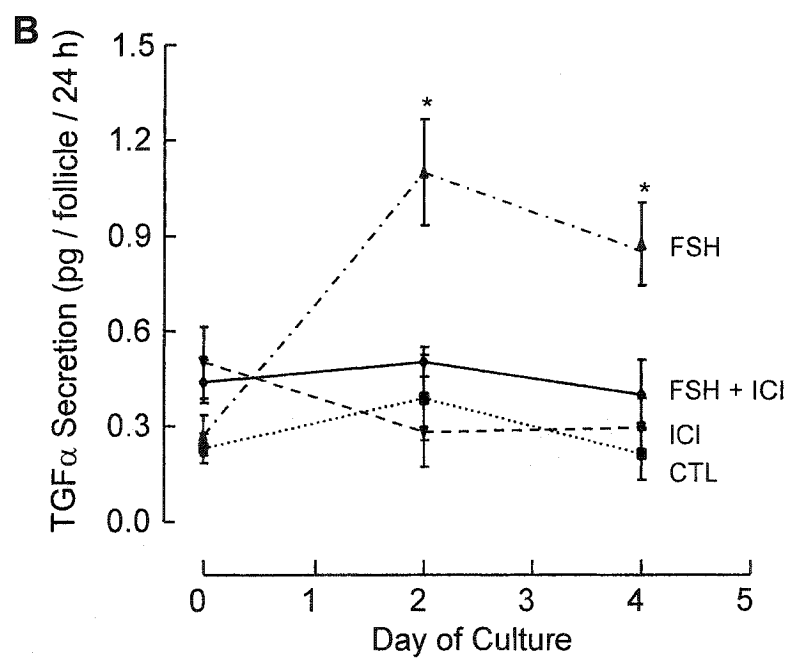
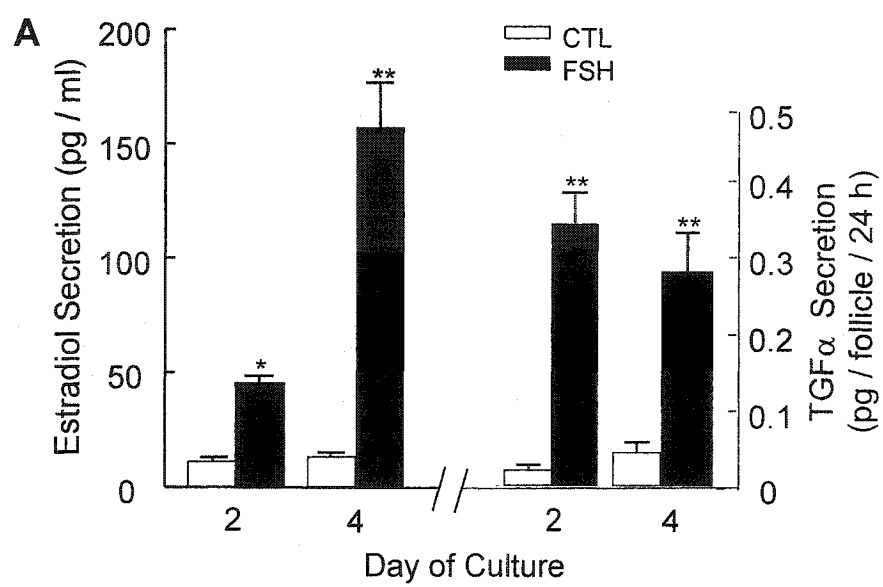
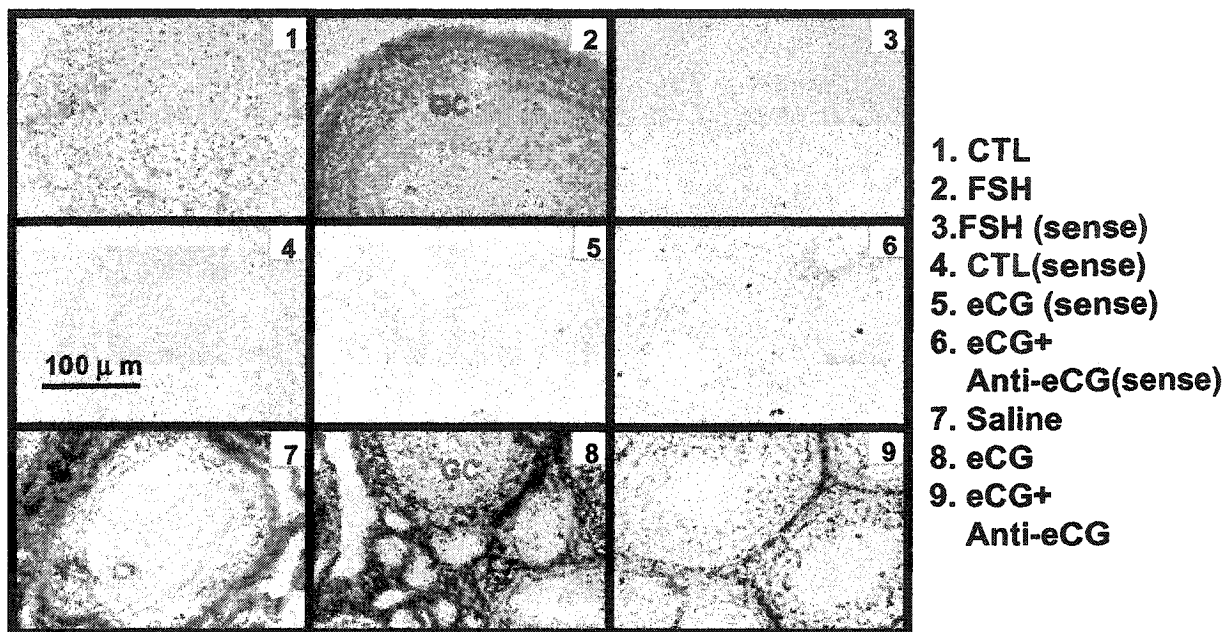


Fig 2

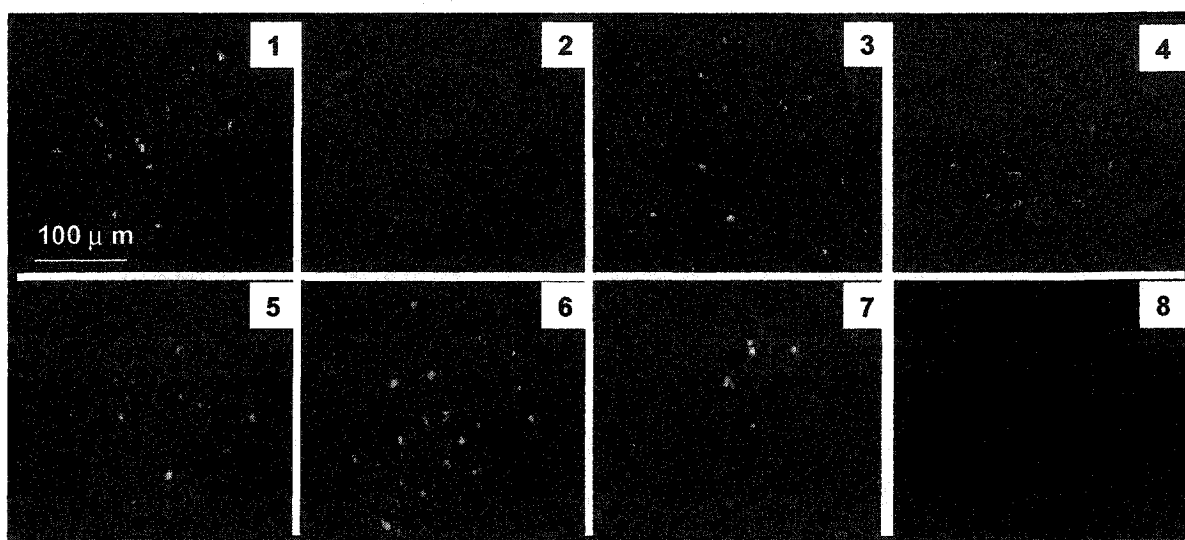
Figure 3. A: In situ hybridization of TGF α mRNA on ovarian or cultured follicle sections.

Ovarian and follicular sections are probed with antisense TGF α cRNA probe [CTL (3A-1), FSH (3A-2), Saline (3A-7), eCG (3A-8), eCG-Anti-eCG (3A-9)] to determine the TGF α mRNA expression. Sense probe was used as negative control [FSH (3A-3), CTL (3A-4), eCG (3A-5), eCG + Anti-eCG (3A-6)]. Six ovaries from 3 rats in each experimental group [saline (3A-7), eCG (3A-8) or eCG + Anti-eCG (3A-9) injected (ip) rats] were embedded into one block. Each in vitro experiment contained six follicles per experimental group cultured in the presence (3A-2) or absence (3A-1) of FSH (100 ng/ml) and were embedded. Sections from three independent experiments were tested. An image from 18 representative ovaries is shown for each treatment group. B: The effects of FSH, E2, TGF α and/or ICI on XIAP expression and apoptosis in rat follicle cultures. Six follicles per experimental group were cultured in the absence (CTL) or presence of FSH (100 ng/ml), E_L (E2 at 5 nM), TGF α (20 ng/ml), ICI (5 nM), FSH + ICI, or FSH (100 ng/ml) + ICI (5 nM) + E_H (E2 at 500 nM) for four days, fixed and embedded into one block and paraffin sectioned. The sections were tested for TUNEL (green) and IHC of XIAP (red) as described in Materials and Methods. Negative control for TUNEL (no TUNEL enzyme in reaction mix) and XIAP (normal rabbit IgG in stead of XIAP antibody) showed undetectable signal indicating minimum non-specific bindings (Fig. 3B-8). An image from 18 representative follicles is shown for each treatment group.

A



B



- | | | | |
|-----------------|--------|-------------------------------|-------------------|
| 1. CTL | 2. FSH | 3. FSH + ICI | 4. E _L |
| 5. TGF α | 6. ICI | 7. FSH + ICI + E _H | 8. NEG |

Fig 3

In the absence of FSH, follicular TGF α mRNA abundance was low although clearly higher than that observed in negative control (with sense probe; Fig. 3, A-1 vs. A-4). Addition of FSH to the follicle cultures markedly increased TGF α transcript levels in both granulosa and theca cells (Fig. 3, A-2 vs. A-1). The signals were more intense in the theca layer (Fig. 3A-2) and readily distinguishable from sections probed with sense cRNA (Fig. 3A-3). Similarly, ovarian sections from eCG-treated rats exhibited intense signal compared to those from saline-treated animals (Fig. 3, A-8 vs. A-7). Signal intensity was also higher in theca than in granulosa cells. Withdrawal of gonadotropin support by anti-eCG treatment *in vivo* resulted in a marked decrease in signal intensity of the TGF α message (Fig. 3A-9 vs. Fig. 3A-8). Signal intensities in the negative control ovarian sections (with sense cRNA probe) were negligible (Fig. 3, A-3 to A-6).

To test if the FSH-stimulated TGF α synthesis is associated with follicular estrogen secretion, spent media from day 2 and day 4 of the follicle cultures were analyzed for E₂. FSH significantly increased E₂ secretion during the four-day culture period ($P < 0.05$; Fig. 2A), with a greater response observable during the latter period. In addition, the addition of the estrogen antagonist ICI (100 nM) to the culture medium, significantly suppressed FSH-induced follicular TGF α secretion ($P < 0.05$; Fig. 2B).

Role of Estrogen in Gonadotropic Regulation of XIAP Expression, Apoptosis, and Follicular Growth In Vitro

To determine the role of estrogen-induced TGF α secretions in the gonadotropic regulation of follicular growth and apoptosis, apoptotic signal (TUNEL) and XIAP immunoreactivity (Immunohistochemistry) were measured in follicles cultured with/without FSH and in the absence or the presence of ICI (100 nM). Follicles cultured in the absence of gonadotropin for 4 days exhibited barely detectable XIAP levels and extensive apoptosis signals (Fig. 3B-1). While addition of FSH to the follicle cultures markedly increased the intensity of the XIAP immuno-

signal and suppressed apoptosis (Fig. 3B-2), the presence of ICI (5 nM) effectively attenuated these responses (3B-3). Similar to FSH treatment, addition of estrogen or TGF α alone to the follicle cultures resulted in increased XIAP expression and suppressed apoptosis when compared to control follicles (Fig. 3, B-4 and B-5, respectively).

To further assess the role of estrogen in the cellular mechanism by which FSH stimulates follicular development in the present culture system, the influence of ICI on FSH-induced follicle growth was studied (Fig. 4A). While ICI alone had minimum or no effect on follicular volume during the four-day culture period, it suppressed the increase in follicular growth in a concentration dependent manner (0-100 nM; $P < 0.01$), with significant response noted at 0.5 nM of the anti-estrogen ($P < 0.05$; Fig. 4A). To test if the above-mentioned ICI effects are estrogen specific, follicles were cultured for four days in medium containing FSH, ICI, E₂ (5 nM) and/or excess E₂ (250 nM and 500 nM [50x and 100x of the concentration of ICI, respectively]) and follicular volume changes (Fig. 4B), TGF α secretion (Fig. 4C), XIAP expression and apoptosis were examined (Fig. 3, B-6 to B-8). Estradiol alone (5 nM) significantly increased the concentrations of TGF α in the spent medium ($P < 0.05$; Fig. 4C) and induced follicular growth ($P < 0.05$; Fig. 4B). While ICI significantly suppressed the FSH-induced TGF α secretion ($P < 0.01$; Fig. 4C) and follicular growth ($P < 0.05$; Fig. 4B), these responses were partially ($P < 0.05$, vs. FSH alone; $p < 0.05$, vs. FSH + ICI; Fig. 4C) and completely ($P = 0.065$, vs. FSH alone; $P < 0.05$, vs. FSH + ICI; Fig. 4B) attenuated by the addition of the excess of E₂, respectively. ICI suppressed FSH-induced XIAP expression and increased TUNEL signal intensity (Fig. 3B-3). Addition of estrogen (500nM) to the culture medium partially reversed these responses (Fig. 3B-7).

Figure 4. Effects of ICI 182780 (ICI) on FSH-induced follicular growth and TGF α secretion.

A: Follicles were cultured in the absence or presence of FSH (100 ng/ml) and different concentrations of ICI (0 – 100 nM) for four days. B & C: The influence of excess estrogen on the suppression of FSH-induced follicular growth and TGF α secretion by ICI during the 4-day culture period. Follicles were cultured with FSH, E_L (E2 at 5nM), FSH + ICI (5 nM, 30 min prior FSH), FSH + ICI + E_M (E2 at 250 nM) or FSH + ICI + E_H (E2 at 500 nM) for four days. Follicle diameter was measured daily. Results are expressed as Mean $\pm$ SEM of three independent experiments each containing 10 follicles per experimental group.

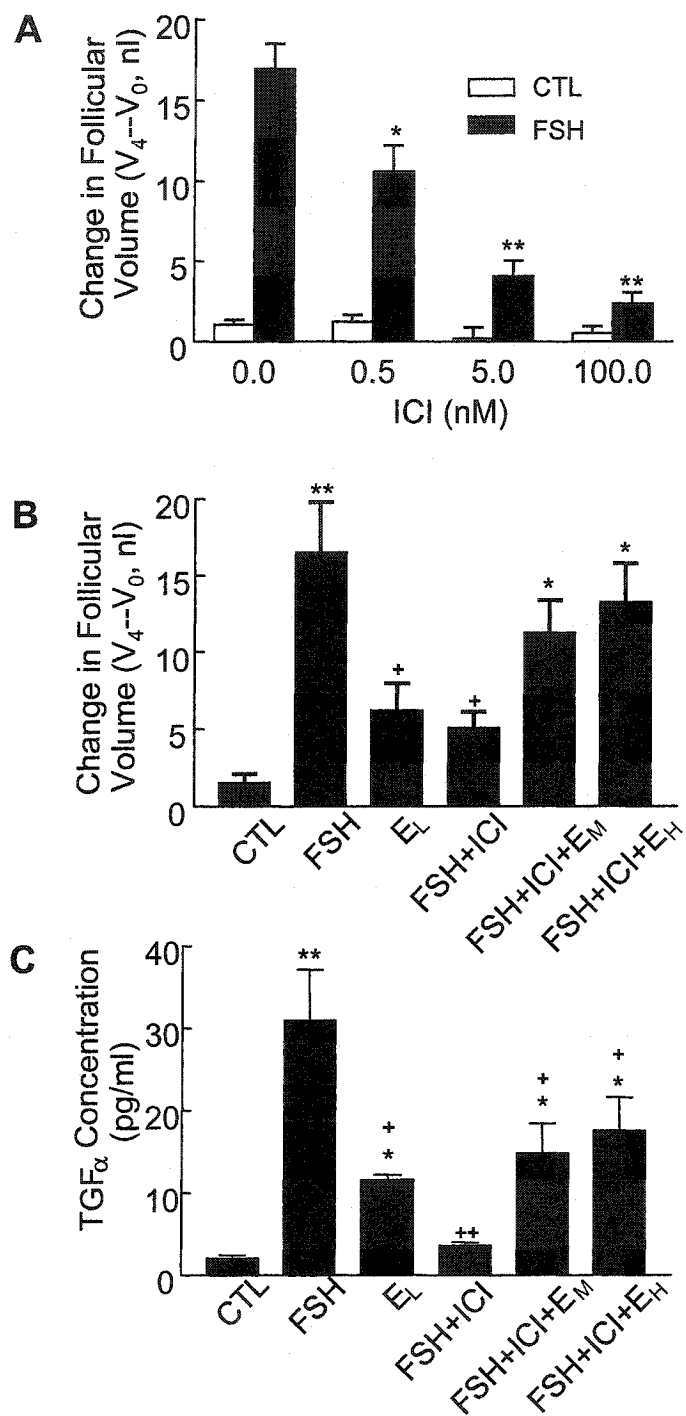


Fig 4

TGF α Alone Up-Regulates Granulosa Cell XIAP Content and Stimulated Proliferation In Vitro

To determine if TGF α alone has a direct effect on XIAP expression and can elicit a mitogenic response, granulosa cells were cultured in the absence or presence of TGF α for 24 h and 48 h respectively. TGF α (0-20 ng/ml) significantly increased granulosa cell XIAP content in a concentration dependent manner ($P < 0.05$; Fig. 5A). Moreover, TGF α (10 ng/ml) significantly increased granulosa cell number ($P < 0.05$; Fig. 5B), a response that was effectively suppressed by the presence of anti-TGF α antibody ($P < 0.05$; Fig. 5B) but not normal IgG (as control).

To determine if XIAP has a role in TGF α -induced granulosa cell proliferation, cells were infected with adenoviral XIAP-antisense cDNA or LacZ (as control) (MOI, 20 and 40) following plating, and cultured with TGF α for an additional 24 h. XIAP down-regulation (as confirmed by Western blot; Fig. 5C) significantly suppressed TGF α -induced [methyl- ^3H] thymidine incorporation into DNA ($P < 0.05$; Fig. 5D).

Figure 5. A: TGF α increases XIAP protein content in granulosa cells. Granulosa cells (0.5 million per 35-mm dish) from eCG-primed immature rats were cultured for 24 h in the presence of different concentrations of TGF α (0-20 ng/ml). Proteins from whole cell lysate were analyzed by Western blot. XIAP content was normalized by α -Tubulin and results are expressed as the fold of control and Arcsine Square root-transformed prior to two-way ANOVA analysis. B: TGF α increases granulosa cell number in vitro. Rat granulosa cells were cultured for two days with or without TGF α plus anti-TGF α antibody or mouse IgG (as control) and cells were counted at the end of culture duration. *: $p < 0.05$ vs. CTL; +: $p < 0.05$ vs. TGF α . C: A representative Western blot image illustrating the changes in XIAP contents following the adenoviral infection (XIAP antisense and LacZ) and TGF α challenge for 24 h. D: TGF α -stimulated thymidine incorporation is suppressed by adenoviral XIAP antisense cDNA infection. Granulosa cells from eCG-primed immature rats were cultured and infected with adenoviral XIAP antisense cDNA or LacZ. Sixteen hours after infection, cells were cultured with or without TGF α for a further 24 h. [3 H]-Thymidine was added 9 h before the end of the culture period. DNA assay and Thymidine incorporation were performed as described in Materials and Methods. [3 H]-Thymidine incorporation is expressed in cpm per μ g of DNA. Results are Mean $\pm$ SEM of three independent experiments.

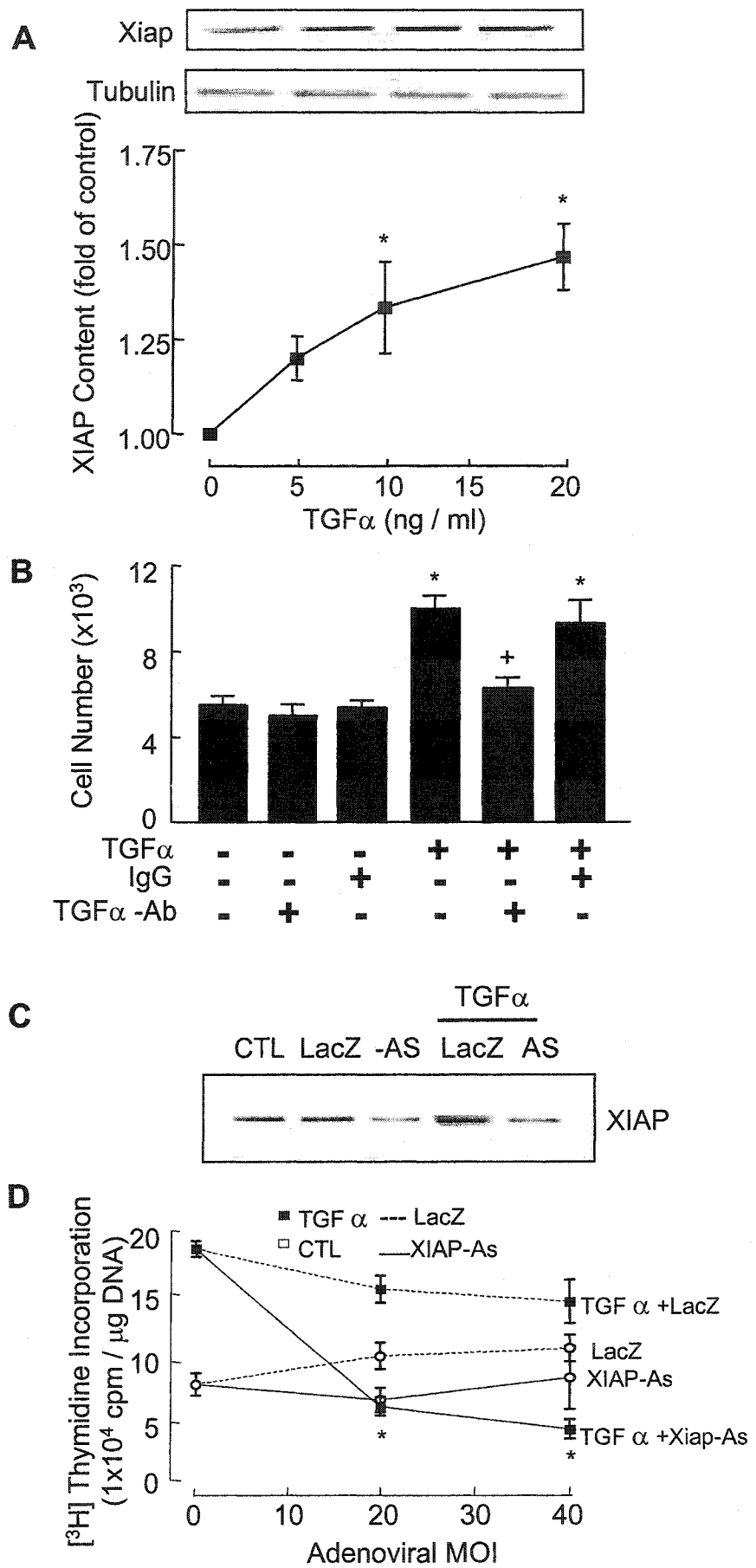


Fig 5

DISCUSSION

The present study demonstrates that FSH stimulates follicular growth and suppresses apoptosis in vitro. In addition, whereas FSH alone failed to elicit a mitogenic response in primary granulosa cell cultures, follicular growth induced by the gonadotropin was attenuated by anti-TGF α neutralizing antibody. These results are consistent with previous findings that follicles cultured without gonadotropin exhibited increased apoptotic DNA fragmentation that was prevented by the addition of FSH or cAMP [28];[29] and also support the concept that TGF α is an important intra-ovarian factor in the gonadotropic regulation of follicular development. In the human, TGF α is present in follicular fluid and its level is inversely correlated with follicle growth, suggesting that importance of TGF α as a mitogenic factor is dependent on follicular stage [30]. TGF α increased [3 H] thymidine incorporation during DNA synthesis in the undifferentiated granulosa cells but not in the differentiated granulosa cells [25].

The regulation of follicular development by FSH is complex and involves the participation of intra-ovarian factors [4]. In this context, it is of interest to note that TGF α alone stimulated follicular growth to a lesser extent compared to FSH, suggesting a possible involvement of other ovarian regulators (e.g. insulin-like growth factor [IGF], EGF, activin, inhibin). Homburg [31] reported that the suppression of apoptosis by FSH is partially mediated through IGF-1. In addition to FSH, EGF and IGF-I can stimulate follicle growth and antrum formation in a bovine preantral follicle culture system [32], and FSH-induced preantral folliculogenesis in the hamster ovary involves EGF gene transcription [33]. Moreover, evidence has shown that other intra-ovarian systems, including those of IGF and activin/inhibin, are also involved in follicle selection in response to gonadotropin [34]. These results illustrate complex cellular interactions in the regulation of granulosa cell fate, and thus also of follicular fate.

It has been demonstrated that follicular atresia in the rat ovary is associated with decreased granulosa cell IAPs contents, and gonadotropin up-regulates follicular IAP expression and stimulates follicular growth in vivo [17]. Whereas granulosa cell IAP level were high during eCG-induced follicular development, gonadotropin withdrawal by anti-eCG antibody decreased granulosa cell IAP content and induced apoptosis. Similarly, FSH has also been shown to increase follicular XIAP expression and growth of rat pre- or early antral follicles in vitro [5]. In the mouse ovary, the expression of NAIP, another member of the IAP family, is also under gonadotropic regulation during follicular development [35]. Increased NAIP gene expression in response to FSH was evident in the granulosa cells of healthy but not atretic follicles. Another IAP, the inhibitor of T-cell apoptosis (ita), is highly expressed in developing but not in prehierachical hen ovarian follicles in vivo [36]. The patterns of ita mRNA expression during follicle development supports the contention that this gene is involved in protecting hen granulosa cells from apoptosis and thus maintaining follicle viability. In the present study, addition of TGF α to the culture medium significantly increased XIAP expression in cultured follicles and follicular growth in vitro. Whereas TGF α also up-regulated granulosa cell XIAP content, down-regulation of XIAP by adenoviral antisense expression markedly attenuated TGF α -stimulated granulosa cells [3 H]-thymidine incorporation in vitro, suggesting that the XIAP may play an important role in the TGF α -mediated gonadotropic stimulation of the follicular growth in the rat ovary.

Precisely how XIAP is involved in the suppression of apoptosis during follicular development is not clear. XIAP is known to modulate receptor-mediated apoptosis by inhibiting caspase-3 [15] and mitochondria-mediated cell death by suppressing caspase-9 activity [37]. Caspase-3 is present in granulosa cells of atretic rat ovarian follicles [38] and the role of Fas-mediated apoptosis has been well established in the rat follicular atresia [19;39]. Whereas tumor necrosis factor (TNF α) is a well-established caspases-3 activator [40], the cytokine failed to

induce granulosa cell apoptosis in vitro unless XIAP was down-regulated by either XIAP antisense expression or co-treatment with the protein synthesis inhibitor cycloheximide [41]. It is conceivable that, like TNF α , FSH up-regulates XIAP expression during follicular development, leading to caspase inhibition and suppressed apoptosis. While the role and gonadotropic regulation of the ovarian Bcl2/Bax expression and the cytochrome C/apoptotic protease-activating factor-1 (Apaf-1) pathway in mitochondria-mediated apoptosis is well established [42;43], FSH has recently been shown to suppress Apaf-1 expression and granulosa cell apoptosis in early antral follicles in vivo [44]. The latter observation raises the interesting possibility that, in addition to up-regulating XIAP to suppress caspases-9, FSH may exert its anti-apoptotic action upstream in the survival pathway. Alternatively, XIAP may play an important role as an anti-apoptotic factor by activating cell survival and/or signaling pathways involved in the regulation of the expression of other cell survival factors. In this context, we have recently demonstrated that up-regulation of XIAP increased phospho-Akt content, a response blocked by the PI3K inhibitor LY294002 suggesting that possibility that XIAP may promote cell survival in part through activation of the PI3K/Akt cell survival pathway [23]. In addition, recent studies have also shown that XIAP is a physiological activator of NF κ B [45], a transcription factor believed to be involved in activation of various cell survival genes, including Flip, Bcl-2 family members, and so on [46-49].

Because granulosa, but not theca, cells have FSH receptor [50], and the theca is the main source of the TGF α (as demonstrated in the present study), the stimulatory action of FSH on TGF α secretion must be mediated through a granulosa cell-derived factor(s). In the present studies, we have confirmed earlier observations that FSH stimulated follicular E₂ production [2] and that theca TGF α /EGF production can be stimulated by this estrogen during follicular development [8;51]. Moreover, we have demonstrated for the first time that, like FSH, both E₂ and TGF α up-regulated the follicular XIAP expression in vitro and that the estrogen antagonist

ICI attenuated FSH-induced follicular TGF α secretion and XIAP expression. These responses appeared to be important for the gonadotropic regulation of follicular growth, since ICI markedly suppressed FSH-induced changes in follicular volume in a concentration dependent manner, a response that could be partially overcome by excess estrogen.

The mechanism by which FSH and TGF α up-regulate granulosa cell XIAP expression is not clear. Previous studies from our laboratory have shown that eCG increases granulosa cell phospho-Akt in vivo and that the phospho-Akt could be co-localized with XIAP. Similarly, it has been demonstrated in various cellular systems that growth factors, such as EGF, can promote cell survival by activating the PI3K/Akt pathway [52]. Whether activation of this pathway in granulosa cells by the gonadotropin during follicular development is mediated through TGF α , however, remains to be investigated.

In conclusion, the present studies have demonstrated that FSH induction of follicular development in vitro is mediated through an estrogen-dependent pathway involving TGF α derived from theca cells. Up-regulation of an anti-apoptotic protein, XIAP, in response to FSH suppresses apoptosis in granulosa cells and facilitates follicular growth (Fig. 6).

Figure. 6 A hypothetical model illustrating the role and regulation of estrogen, TGF α , and XIAP expression involved in granulosa cell (GC)-theca cell (TC) interaction during follicular development following FSH stimulation. The estrogen antagonist ICI 182780 (ICI) and anti-TGF α neutralizing antibody were used in the present study to test the dependence of these intra-ovarian factors in this process.

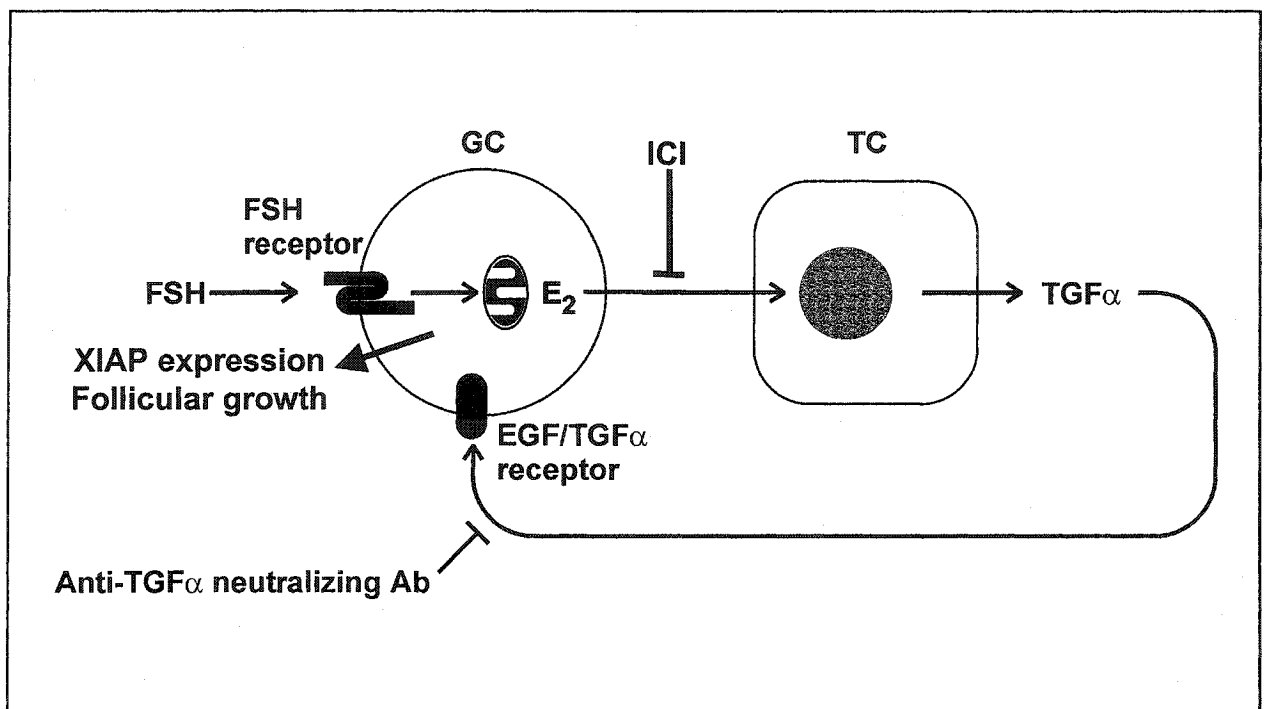


Fig 6

ACKNOWLEDGEMENTS

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CHAPTER 4 ENDOCRINOLOGY 143:2732-2740, 2002

Involvement of Inhibitory Nuclear Factor- κ B (NF κ B)-independent NF κ B Activation in the Gonadotropic Regulation of X-Linked Inhibitor of Apoptosis Expression during Ovarian Follicular Development *in vitro*

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Abstract

Increased XIAP expression and suppressed follicular apoptosis are important determinants in the regulation of follicular development by FSH. The objective of the present study was to examine the role and regulation of nuclear factor- κ B (NF κ B) in the gonadotropic control of granulosa cell XIAP expression and follicular growth *in vitro*. FSH (100 ng/ml) increased rat granulosa cell XIAP mRNA abundance and protein content. The gonadotropin also induced granulosa cell p65 subunit-containing NF κ B translocation from cytoplasm to nucleus and increased NF κ B-DNA binding activity. Super-shift EMSA indicated the FSH-activated NF κ B contained p65 and p50 subunits. Unlike TNF α , FSH failed to elicit a significant change in granulosa cell phospho- and total- inhibitory NF κ B (I κ B) contents *in vitro* and dominant negative I κ B expression was ineffective in blocking the increase in NF κ B-DNA binding activity and XIAP protein content induced by the gonadotropin. In contrast, SN50 (a cell permeable inhibitory peptide of NF κ B translocation; 50-200 ng/ml) suppressed FSH-stimulated NF κ B-DNA binding, XIAP expression and follicular growth. FSH also increased granulosa cell phospho-Akt contents, a response sensitive to the PI3K inhibitor LY294002 (10 μ M) and Wortmannin (100 nM). In conclusion, the present studies demonstrate that the FSH-induced XIAP expression is mediated through the NF κ B pathway via activation of PI-3 kinase rather than the classical I κ B kinase.

Introduction

The presence of FSH is critical for the selection and survival of ovarian follicles during development (1-4). The gonadotropin has been shown to stimulate rodent ovarian follicular growth and to facilitate pre-antral follicle development induced by IGF-I *in vitro* (5-8). In addition, FSH is an apoptosis suppressor for granulosa cells *in vitro* (9;10). It is well established that the acquisition of FSH receptor(s) during follicular growth and their coupling to signaling pathways are key events in follicular development and dominance. Although cAMP is a well-established second messenger of FSH, other signal pathway(s), such as those of calcium/calmodulin and phospholipase C/protein kinase C/inositol 1,4,5-triphosphate, have also been suggested to play a role in gonadotropin action (11-13). The recent discovery of different isoforms of FSH receptor (i.e. FSH-R3), which result from hormone-induced receptor gene splicing (14) raises the possibility that FSH may be mediated through different receptors and signaling pathways. Furthermore, studies from our laboratory have demonstrated that equine chorionic gonadotropin (eCG) increases rat ovarian follicular phospho-Akt content *in vivo* (15), a response sensitive to the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002. Whether this response is a consequence of the direct action of gonadotropin or mediated through other ovarian factors is unclear.

The inhibitor of apoptosis (IAP) family, first identified in baculovirus, includes X-linked IAP (XIAP or cIAP-3), human IAP-1 (HIAP-1 or cIAP-2), human IAP-2 (HIAP-2 or cIAP-1), neuronal apoptosis inhibitory protein (NAIP), Survivin and Livin (16-18). Although only a few reports to date have addressed the subcellular action of these anti-apoptotic proteins, XIAP, HAIP-1 and HAIP-2 have been shown to be direct

inhibitors of caspase-3 and caspase-7 (19) and to modulate the Bax/cytochrome C pathway by inhibiting caspase-9 (20). In the ovary, XIAP is up-regulated by gonadotropin and necessary for follicular development *in vivo* (21) and *in vitro* (7). Preliminary studies have shown that while down-regulation of XIAP by antisense expression induces follicular cell apoptosis, XIAP over-expression suppresses cell death (22). Although these findings indicate that XIAP plays an important role in follicular cell survival, the signaling mechanism(s) involved in the gonadotropic regulation on XIAP expression is unclear.

Nuclear factor κ B (NF κ B) is a group of inducible dimeric transcription factors. They are composed of DNA-binding proteins (Rel) that recognize a common sequence motif on the NF κ B-regulated genes. It has been demonstrated that NF κ B activation increases the expression of genes involved in the inflammatory response (23) and prevention of TNF α -induced apoptosis, such as zinc finger protein A20 (24), and members of the Bcl-2 (25) and IAP (26) families. Other studies have shown that *XIAP* and *HIAP-1* have NF κ B-binding motif at their 5' end non-translational regions, and that IAP expression is NF κ B activation-dependent (27-29). If and how gonadotropic regulation on XIAP expression is mediated through the NF κ B pathway is unknown.

Delfino and Walker (30) reported that FSH increases NF κ B-DNA binding activity in rat Sertoli cells. Previous studies from our laboratory have demonstrated that TNF α increases rat granulosa cell XIAP content via the NF κ B pathway *in vitro* (29). However, if and how NF κ B activation plays a role in the gonadotropic control of the granulosa cells fate (survival verses apoptosis) during follicular development has not been investigated. The objective of the present study was to assess the possible involvement of the NF κ B

pathway in the gonadotropic regulation of granulosa cell XIAP expression and follicular growth *in vitro*.

Materials and Methods

Materials

Culture media and fetal bovine serum (FBS), Trizol, deoxynucleotide triphosphate (dNTP) and Muloney murine leukemia virus reverse transcriptase (RT) were purchased from Gibco Bethesda Research Laboratories (Burlington, ON, Canada). Oligo dT and RNase inhibitor were products of Ambion Inc. (Austin, Texas, USA). HotStarTaq DNA polymerase, RNeasy mini kit, PCR purification kit and the Effectene transfection reagent were from Qiagen Inc. (Mississauga, ON, Canada). Low-melting-point agarose (low melting point agarose), Triton X-100, Tween 20, eCG, Tris, collagenase, Ponceau S, DNase 1 and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma (St. Louis, MO). The chemiluminescence ECL Western blotting detection kit, and [$\gamma^{32}\text{P}$]-ATP (30 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Ovine FSH (NIAMDD oFSH-14) was obtained from NIDDK (Baltimore, MD). Nitrocellulose membrane, acrylamide (electrophoresis grade), *N*, *N'*-methylene-bis-acrylamide, ammonium persulfate, dithiothreitol (DTT), glycine and Bio-Rad protein assay kit, SDS-PAGE prestained molecular weight standards (low range), and anti-rabbit and anti-mouse IgG-horseradish peroxidase conjugated products were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). X-ray films were from Eastern Kodak Company (Rochester, NY). Chamber slides were from Nunc Inc. (Naperville, IL). Recombinant human TNF α was from R&D Systems Inc. (Minneapolis, MN). SM50 and SN50 were from BioMol Research Laboratories, Inc. (Plymouth Meeting, PA). NF κ B-oligo probe and T4 polynucleotide kinase were from Promega Corp. (Madison, WI). Fluoresce

expression vector pcDNA3.1/CT-GFP, parental vector pCMV and pCMV-I κ B construct containing dominant negative I κ B α (serine-to-alanine mutation at residue 32 and 36) were from CLONTECH Laboratories, Inc. (Palo Alto, CA). Multiwell plates were from Becton Dickinson Labware (Franklin Lakes, NJ). Rabbit polyclonal antibodies against human phosphorylated and total I κ B- α were from Santa Cruz Biotechnology (Santa Cruz, CA); XIAP antibody was a generous gift from Dr. Eric LaCasse, Aegera Therapeutics Inc. (Ottawa, ON, Canada). P65 shift Kit was from Geneka Biotechnology Inc. (Montreal, PQ, Canada). NE-PER nuclear and cytoplasmic extraction reagents were from PIERCE Biotechnology (Rockford, IL).

Animal preparation and culture of rat ovarian granulosa cells

Immature female Sprague Dawley rats (24-25 d old) from Charles River Laboratories, Inc. Canada (Montreal, Quebec, Canada) were injected with eCG (15 IU, ip) and ovaries were collected 24 h thereafter in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with HEPES (10 mM, pH 7.4) and FBS (10%). Granulosa cells were harvested by follicle puncture as previously described (31), washed and centrifuged (900 $\times$ g, 10 min). Cells were plated for 24 hr in RPMI 1640 medium with FBS (10%) under a humidified atmosphere of 95% air and 5% CO₂ and cultured in serum-free medium containing FSH, TNF α , SN50 (SM50 as control) for various duration.

Transient transfection

Rat granulosa cells were seeded in 60-mm dishes (1×10^6 cell/dish) and transfected the following day with either 4 μ g of the expression vector pcDNA3.1/CT-GFP, pCMV or pCMV containing mutated I κ B, using the Effectene transfection reagent. Twenty-four hours after transfection, cells were treated with FSH (100 ng/ml) or TNF α (20 ng/ml) for 30 minutes and then harvested for further analyses. Transfection efficiency, defined as the percentage of pcDNA3.1/CT-GFP transfected cells with fluorescent signal, was 30%.

Follicular isolation and culture

Ovaries from 22- to 24-d-old rats were cut into small pieces and incubated (37 C, 30 min) in α -MEM medium containing collagenase (Type 1A, 4 mg/ml) and DNase 1 (0.3 mg/ml). The incubation was terminated with the transfer of the ovarian tissues into Leibovitz's L-15 medium with BSA (0.1%) and follicles (160-210 μ m) were dissected using 28 $^{1/2}$ -gauge needles. To minimize the experimental variation due to damage incurred during the isolation procedures, only follicles judged to be normal (with oocyte and granulosa cells completely enclosed by the basement membrane and the theca layer) were selected for experiments on both the day of isolation (d 0) and on d 1 of culture (7). Confocal microscopic (M500; Bio-Rad Laboratories Ltd., Hertfordshire, UK) examination of the selected follicles [following fixation with paraformaldehyde (4 %; 30 min, RT) and staining with ethidium bromide (5 mg/ml; 15 min, RT)] revealed that they were at the preantral (75%) and early antral (25%; as evident by the presence of an antral space as large as an area occupied by about 3 granulosa cells) stages of development. Selected follicles were cultured individually for 6 days in 96-well plates in 100 μ l of

follicular culture medium [α MEM medium supplemented with HEPES (10 mM), BSA (0.1%), rat serum (1 %), bovine insulin (5 μ g/ml), transferrin (10 μ g/ml), ascorbic acid (25 μ g/ml), sodium selenium (1 ng/ml) (32), non-essential amino acids (1%), streptomycin-penicillin (0.5%) and fungizone (0.25%)] with or without FSH. The follicular size was determined daily before the medium change during the 6 day culture duration. The changes in follicular volume were defined as volume difference between day n and d 0 ($V_n - V_0$, nl).

Protein extraction and Western blot analysis

Changes in protein [XIAP, total- and phospho-inhibitory NF κ B (T-I κ B, p-I κ B), total- and phospho-Akt (T-Akt, p-Akt)] contents were assessed by Western blot as previously described (21). Granulosa cells attached and detached from the growth surface were harvested (0.25% trypsin, 37 C, 3 min) and pelleted. Whole-cell lysate was extracted by addition of ice-cold lysis buffer [PBS, Nonidet P-40 (1%; vol/vol), sodium deoxycholate (0.05%; wt/vol), SDS (0.1%; wt/vol)] containing protease, phosphatase and kinase inhibitors [PMSF (10 μ M), aprotinin (50 μ g/ml), sodium orthovanadate (1 mM), sodium pyrophosphate (Nappi, 10 mM), leupeptin and pepstatin (both 5 μ g/ml)]. Nuclear and cytoplasmic fractions from granulosa cells were prepared with the NE-PER kit, according to manufacturer's instructions. Cells were sonicated (5 sec/cycle, 3 cycles; 0 C), incubated on ice (30 min), and centrifuged (15,000 x g; 30 min.). The sonicates were pelleted and the supernatant was retained and stored at -20 C. Protein content of the extracts was determined with the DC protein assay reagent (Bio-Rad Laboratories, Inc.). Samples were mixed with loading buffer, boiled for 5 min to denature proteins, resolved

by 10% SDS-PAGE and electrotransferred (30 V, overnight) onto nitrocellulose membranes. Each membrane was stained and scanned for total protein with Ponceau S (0.2%) before immuno-blotting. After blocking 1 h in 5% Blotto [Tris-HCl (10 mM; pH 8.0), NaCl (150 mM), Tween-20 (0.05%; vol/vol) (TBS-T) containing skim milk (5%; wt/vol)], membranes were incubated with primary antibodies (RT, 3 h; or 4 C, overnight) in TBS-T containing 5% nonfat milk, and subsequently with horse radish peroxidase-conjugated secondary antibody (1:5000~10,000) in TBS-T with 5% milk (RT, 45 min). Peroxidase was visualized with the ECL kit according to the manufacturer's instructions. The signal of specific protein (e.g. XIAP, Akt, p-Akt, I κ B, and p-I κ B) was scanned and determined by dividing its signal intensity by that of the corresponding total protein to correct for any loading difference between lanes. The intensity of protein of interest was densitometrically determined, using Molecular analyst software version 1.4 (Bio-Rad Canada, Mississauga, ON), and expressed as fold of control prior to statistical analyses and presentation.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts of rat granulosa cells were prepared as previously described but with minor modifications (33). Briefly, 3×10^6 cells were pelleted (200 x g; 5 min) and resuspended in 30 μ l of cold buffer A [HEPES (10 mM), pH 7.9, KCl (10 mM), MgCl₂ (1.5 mM), DTT (0.5 mM), PMSF (0.5 mM), Nonidet P-40 (0.67%)]. Cells were vortexed (15 sec) and placed on ice to swell (15 min), and then centrifuged (16,000 x g, 4 C, 20 min). The supernatant was collected and stored at -80 C. The cell pellet (containing cell nuclei) was resuspended in 30 μ l of buffer B [HEPES (20 mM), pH 7.9, NaCl (0.4 M),

EDTA (0.2 mM), MgCl₂ (1.5 mM), DTT (0.5 mM), PMSF (0.5 mM)] and vortexed vigorously (4 C, 15 min). The nuclear extract was centrifuged (10,000 x g, 30 min) and stored at -80 C. Double-stranded DNA oligonucleotides containing consensus sequences for NFκB binding site was ³²P-labeled with [γ-³²P]-ATP and T4 polynucleotide kinase. Nuclear proteins (8 μg) were incubated with radiolabeled DNA probes (RT, 20 min) in the binding buffer. For supershift assays, nuclear proteins were incubated (RT; 1h) with anti-mouse p65 and p50 polyclonal antibodies (4 μg) before the addition of the labeled DNA probes. Nuclear acid-protein complexes were resolved on a native 5% polyacrylamide gel in Tris-buffered EDTA (1x; pH 8.0) and detected by autoradiography.

Quantification of XIAP mRNA by RT-PCR

Rat granulosa cells were plated in RPMI-1640 with 10% FBS for 24 h and subsequently incubated with FSH at various concentrations (25-100 ng/ml) for another 6 h. Total RNA was isolated from cultured cells with Trizol reagent or RNeasy mini kit, according to manufacturer's instructions. One microgram total RNA was reverse transcribed for cDNA synthesis, using oligo-dT as primer. One tenth of the cDNA synthesized was then amplified with the following primers: rat XIAP [forward: 5'-GGTGGACAAGTCCTATTTTCAA-3' (228-249), reverse: 5'-TCCTGATTACTTAAAGTCGATTCACA-3' (628-602)]; β-actin [forward: 5'-GAAACTACCTTCAACTCCATC-3', reverse: 5'-CGAGGCCAGGATGGAGCCGCC-3']. The samples were denatured (95°C for 15 min), amplified for 30 cycles (XIAP) or 25 cycles (β-actin) at 94 C for 45 s, 56 C for 1 min, and 72 C for 1 min, with the last cycle at 72 C extended for 15 min. Different number of cycles (15, 20, 25, 30 and 35) was tested

for XIAP RT-PCR, and 25 and 30 cycles were found to produce PCR products in the linear range of the analysis. Samples were resolved on a 2% agarose gel and visualized with ethidium bromide. The fluorescent image of ethidium bromide-stained PCR products were captured using the BioRad Gel Doc 1000 system (Bio-Rad Laboratories, Inc.) and densitometrically quantified by the Molecular Analyst Program (version 1.4). XIAP mRNA abundance was normalized against its respective β -actin mRNA and expressed as fold of control prior to statistical analysis.

Immunocytochemistry

Rat granulosa cells, cultured for 6 h on chamber slides in the absence and presence of FSH (100 ng/ml), were fixed (RT, 20 min) with paraformaldehyde (4 %), permeabilized with NP-40 (0.5%) in PBS [NaCl (137 mM), Na_2HPO_4 (8.10 mM), KCl (2.68 mM), KH_2PO_4 (1.47 mM)] and quenched in ammonium acetate (50 mM). They were incubated with monoclonal p65 antibody (1:50) in PBS containing bovine serum albumin (0.1 %; RT, 1 h) and subsequently with HRP-conjugated secondary antibody (RT, 1h) and stained with DAB mix.

Statistical analysis

Results are expressed as the mean $\pm$ SEM of three experiments. Statistical analysis was carried out by one- or two-way ANOVA. Ratio data (mRNA or protein content defined as fold of control) were Arcsine square root transformed prior to ANOVA. Significant differences between treatment groups were determined by the Tukey test. Statistical significance was inferred at $P < 0.05$.

Results

FSH significantly increased both granulosa cell XIAP mRNA abundance and protein content ($P < 0.01$; Fig. 1A) in a concentration-dependent manner, reaching a plateau at 100 ng/ml. Since SN50 is known to bind to the nuclear localization signal (NLS) of NF κ B and consequently blocked its translocation from the cytoplasm to nucleus (34), the influence of this cell permeable peptide on XIAP expression induced by FSH was assessed (Fig. 1B). Pretreatment of the cells with SN50 resulted in a marked decrease in FSH-induced XIAP expression, whereas the control peptide SM50 was ineffective. Two-way ANOVA indicates a significant concentration and treatment effects on XIAP mRNA abundance ($P < 0.01$ for both) and protein content ($P < 0.05$ and $P < 0.001$, respectively), as well as a significant interaction between these factors in the message ($P < 0.05$) and protein ($P < 0.01$) levels. In the presence of SN50, the decrease in XIAP protein content appeared to be greater than that in mRNA abundance ($P < 0.05$; Fig. 1B).

To confirm the involvement of NF κ B translocation in this process, the subcellular localization of p65 subunit-containing NF κ B was assessed by immunocytochemistry (Fig. 2) and cell fractionation-Western blotting (Fig. 3), using an anti-p65 antibody. While p65 was predominantly found in cytoplasm in control cells, addition of FSH to the granulosa cell incubations markedly increased the immunoreactivity of p65 in the nucleus. SN50, but not its control peptide SM50, effectively blocked this response (Fig 2). Western blot on nuclear and cytoplasmic extracts from cells treated for different durations with FSH (Fig. 3) indicates that the gonadotropin induces a time-dependent and concomitant changes in p65 contents in cytoplasmic ($P < 0.05$) and nuclear fractions ($P <$

Fig 1 *In vitro* effects of FSH and SN50 on granulosa cell XIAP mRNA abundance and protein content. A: Rat ovarian granulosa cell from eCG-primed immature rats were cultured in the presence of FSH (0-200 ng/ml). B: Granulosa cells were pretreated with SN50 (cell permeable NF κ B translocation inhibitor) or SM50 (mutated inactive peptide, as control) and cultured with FSH (100 ng/ml). XIAP mRNA (6 h) and protein content (24 h) were determined by RT-PCR and Western blot, respectively. Representative images (top) and densitometric analysis (bottom; expressed as fold of control) are shown. Mean $\pm$ SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared with control.

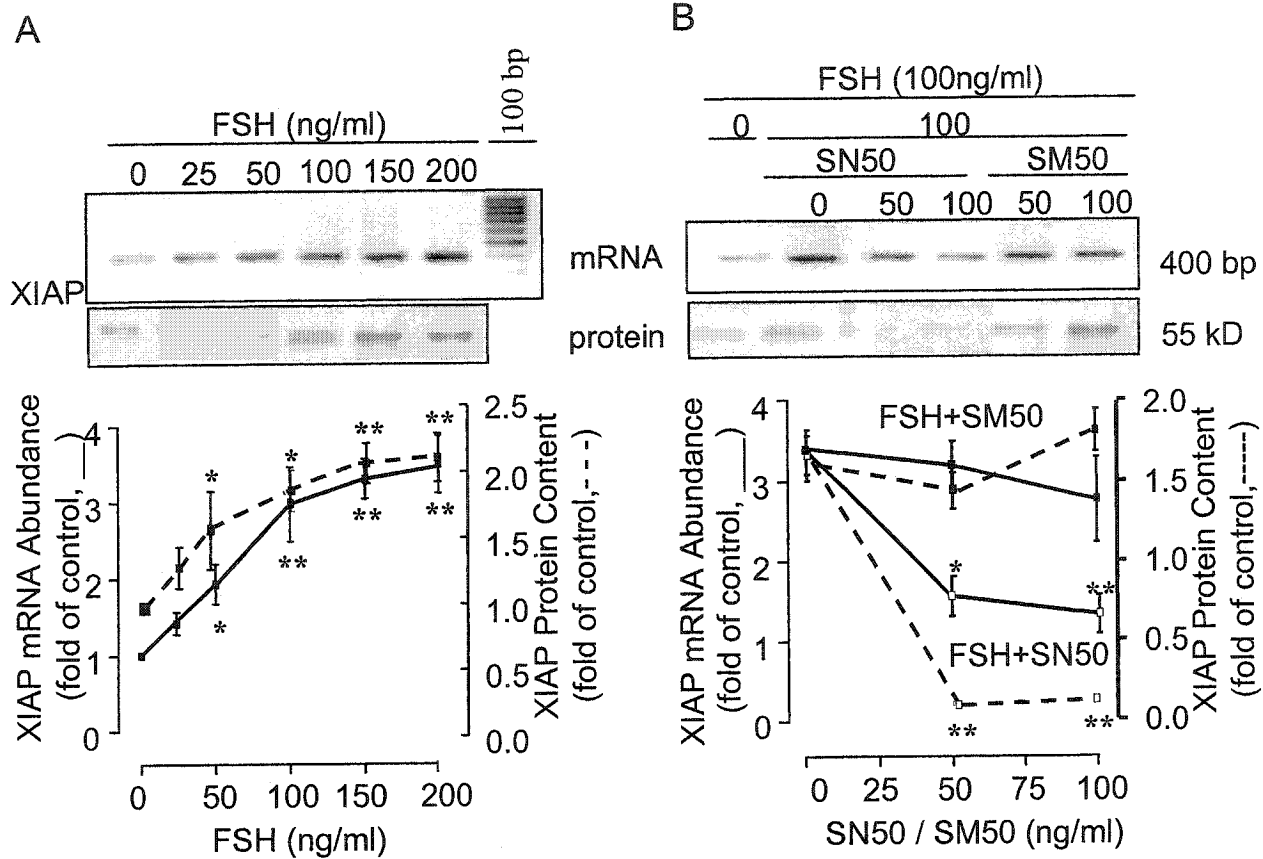


Fig 1

Fig 2 FSH-induced p65 containing-NF κ B translocation. Granulosa cells were incubated with or without FSH, FSH + SN50 or FSH + SM50 for 30 min in chamber slides. Cells were fixed by 10%-neutralized formalin, probed with anti-p65 antibody and stained with DAB. Three independent experiments were performed and represented images are shown. N: nucleus; C: cytoplasm.

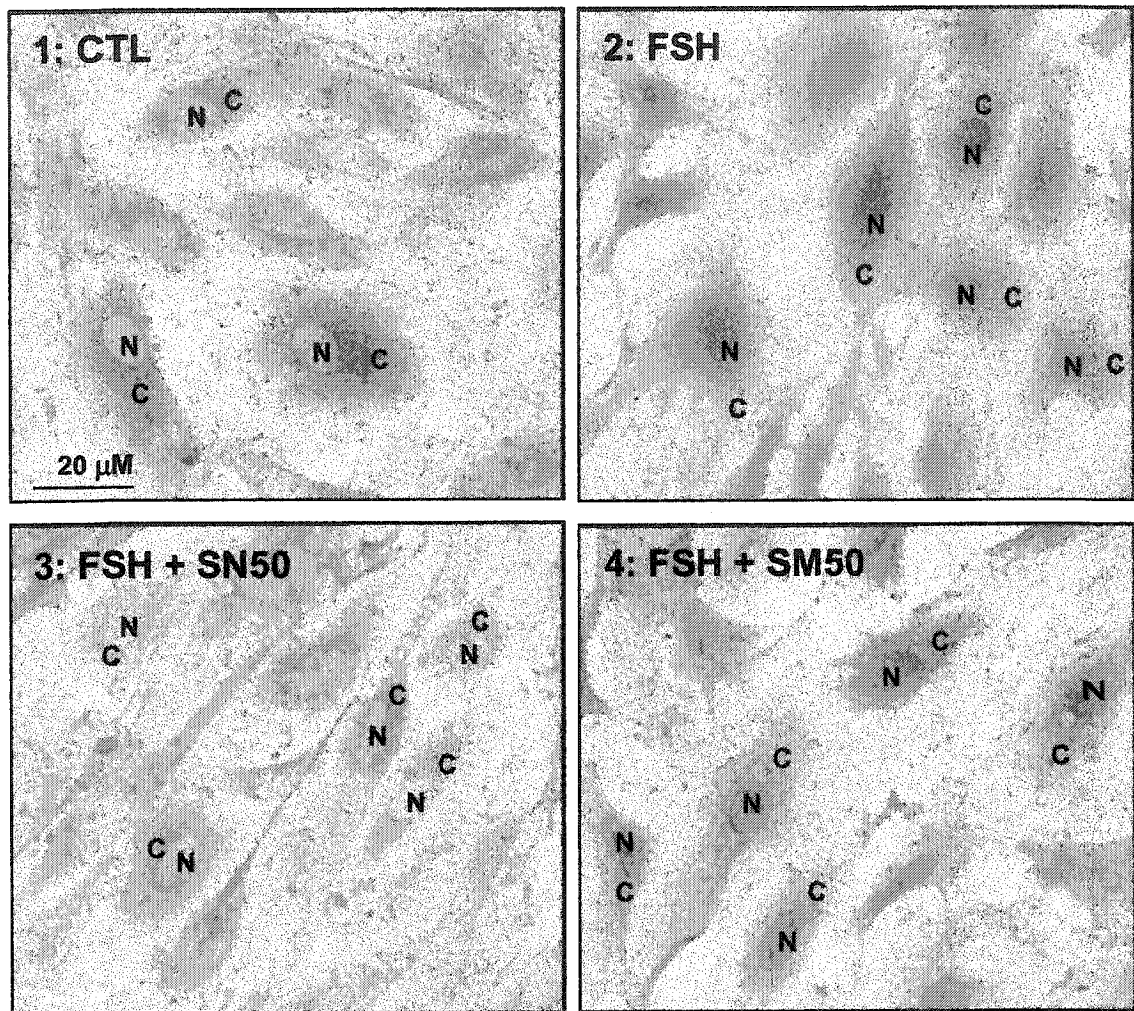


Fig 2

Fig 3 Effects of FSH on changes in p65 contents in granulosa cell cytoplasmic and nuclear extracts. Granulosa cells were incubated for varies duration (0-30 min) with FSH (100 ng/ml). Proteins from cytoplasmic and nuclear fractions were extracted and p65 contents were measured by Western blot. Representative images (top) and densitometric analysis (bottom) of p65 contents (normalized against total protein and expressed as fold of control) are shown. Mean $\pm$ SEM of three independent experiments. *, $P < 0.05$ compared with control.

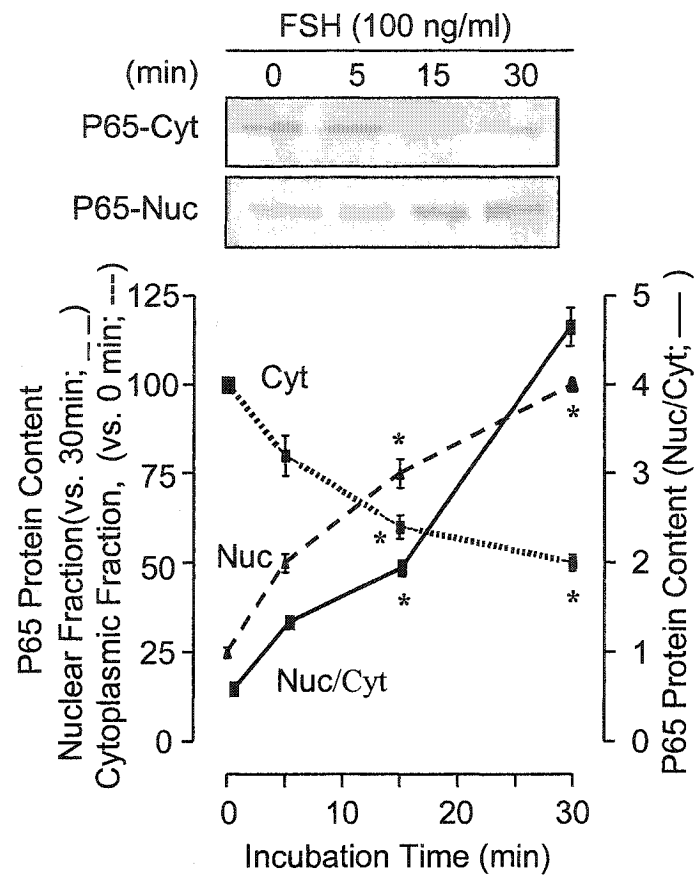


Fig 3

0.01), resulting in a rapid increase in the nuclear/cytoplasmic ratio of the protein ($P < 0.01$). Taken together, the above findings suggest that FSH-induced granulosa cell XIAP expression is mediated through NF κ B translocation from cytoplasm to nucleus.

To determine if FSH-induced translocated NF κ B is indeed biologically active, the effects of FSH on NF κ B-DNA binding activity were tested by EMSA (Fig. 4). FSH increased the binding of NF κ B to DNA in a concentration- (Fig. 4A) and time-dependent (Fig 4B) manner, with a maximum and earliest observable response at 100 ng/ml and 10 min, respectively. When granulosa cells were pretreated with SN50 or SM50 (50-200 ng/ml) before the gonadotropin challenge, the increased NF κ B-DNA binding activity was suppressed by the active peptide (but not by SM50) in a concentration-dependent manner. Incubation of the nuclear extracts with anti-p65 or p50 antibody prior to EMSA resulted in the appearance of slower mobility signals (Supershift; Fig. 4D). In contrast, incubation of the nuclear extracts with IgG (as negative binding control) was ineffective and resulted in NF κ B-DNA binding activity similar to that observed with extracts incubated without antibody or IgG (control). These findings are consistent with the hypothesis that up-regulation of XIAP by FSH is mediated through the activation of NF κ B, which involves the p65 and p50 subunits.

It is well established that NF κ B activation is mediated by I κ B kinase (IKK) activation and subsequent I κ B-phosphorylation and degradation (34). To test if the FSH-induced NF κ B activation is also I κ B-dependent, changes in granulosa cell total I κ B and phospho-I κ B contents following FSH challenge as well as the influence of a dominant negative I κ B on NF κ B activation and XIAP content were assessed. FSH (100 ng/ml) had no effects on both p-I κ B and total I κ B contents during the experimental period (0-30

Fig 4 Concentration (A) and time course (B) studies on the influence of FSH on NFκB-DNA binding activity. Granulosa cells were cultured in the absence and presence of FSH (A, 50-200 ng/ml, 30 min; B, 100 ng/ml, 0 to 30 min). Nuclear proteins were extracted and NFκB binding activity was assessed by EMSA. Effects of SN50 on FSH-stimulated NFκB-DNA binding activity (C) and p65 and p50 subunits involved in FSH-activated NFκB based on supershift assay (D). Granulosa cells were exposed to different concentration (0-200 ng/ml) of SN50 or SM50 (as control) for 15 min prior to FSH challenge (100 ng/ml) and were incubated for an additional 30 min. Nuclear proteins were extracted and NFκB binding activity was assessed by EMSA. Representative images (top) and densitometric analysis (bottom) of NFκB binding activity (as fold of control) are shown. The subunits of the FSH-activated NFκB were assessed by super shift EMSA, using p65 and p50 antibodies, and IgG in the place of an irrelevant antibody. CTL represents sample not incubated with any antibody. Arrow indicates the supershift bands. Mean ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared with control.

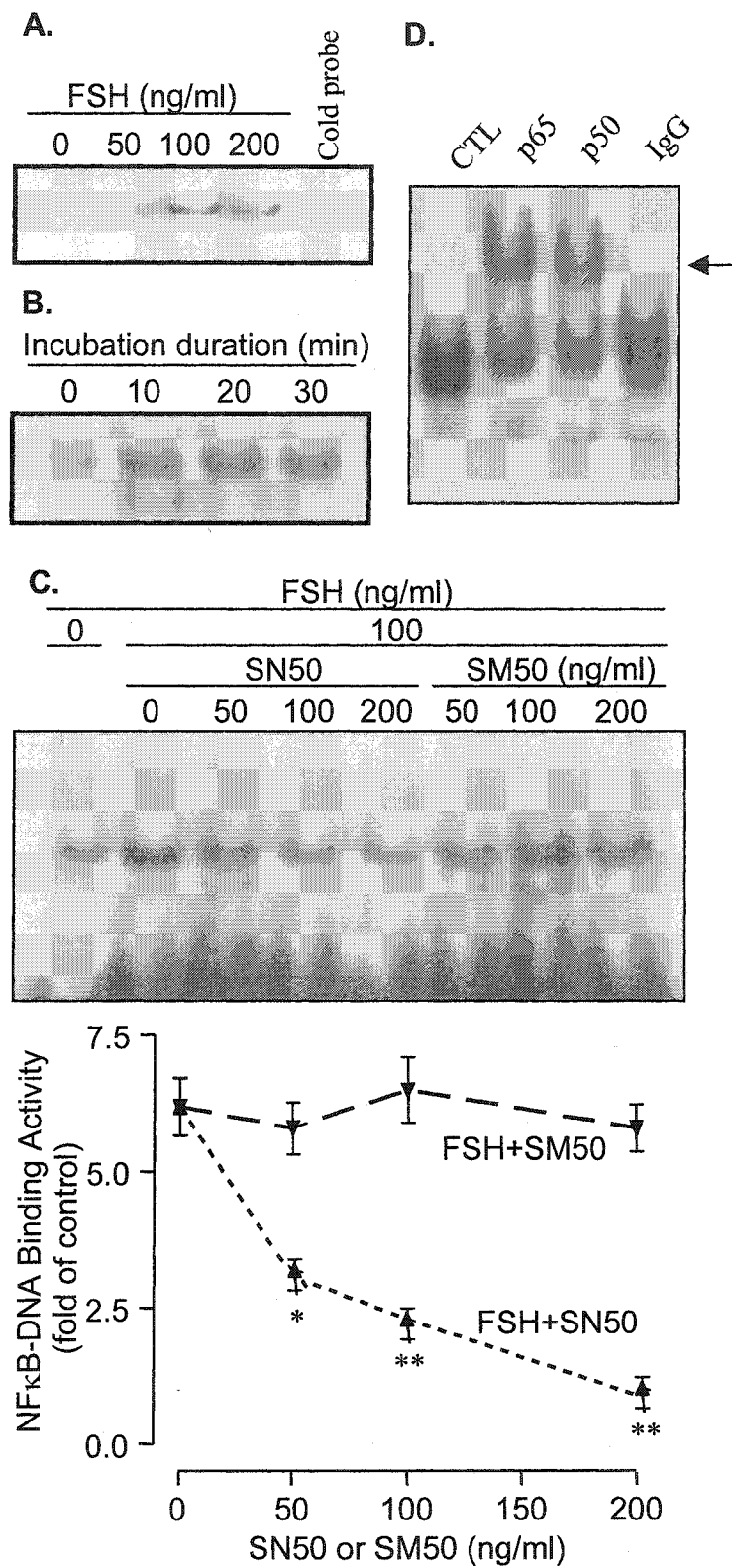


Fig 4

min; Fig. 5A). In addition, over-expression of the dominant negative I κ B (as confirmed by T-I κ B Western blot) failed to suppress the FSH-induced NF κ B-DNA binding activity and XIAP expression (Fig. 5B). In contrast, TNF α (20 ng/ml), an established inducer of IKK-mediated NF κ B activation ($P < 0.01$, Fig 5B), markedly increased p-I κ B level and decreased total I κ B content after 5 and 15 min of exposure, but with both responses returning to basal level at 30 min (Fig. 5A). Whereas extracts from granulosa cells transfected with the mutated I κ B construct suppressed TNF α -induced NF κ B-DNA binding activity and XIAP contents ($P < 0.01$ and $P < 0.05$, respectively, Fig. 5B), the dominant negative I κ B has no significant effect on these responses induced by FSH.

Gonadotropins have been shown to activate granulosa cell PI3K / Akt pathway *in vivo* (15) and *in vitro* (35). PI3K / Akt signaling pathway is known to play a role in IGF-1-mediated granulosa cell survival (36;37), oocyte maturation (38;39) and fetal oogenesis (40). IGF-1 and TGF α induce rapid Akt phosphorylation in hen granulosa cells of preovulatory follicles, the inhibition of which by the PI3K inhibitor LY292004 leads to apoptosis (41). To determine if this signal pathway is involved in the FSH-induced NF κ B activation, the influence of FSH on phospho- and total-Akt contents (Fig. 6) as well as the effect of the PI3K inhibitor LY292004 (Fig. 7A) or Wortmannin (Fig. 7B) on FSH-induced NF κ B-DNA binding activity and XIAP protein content were assessed. Concentration-response studies indicate that, while ineffective on granulosa cell total Akt content, FSH significantly increased phospho-Akt levels ($P < 0.05$; Fig. 6). Pretreatment of granulosa cells with LY292004 (1-20 mM) or Wortmannin (25-150 nM) prior to FSH (100 ng/ml), suppressed the FSH-activated NF κ B-DNA binding activity ($P < 0.01$) and XIAP content ($P < 0.01$) in a concentration-dependent manner (Fig. 7).

Fig 5 Influence of TNF α and FSH on phospho-I κ B, total I κ B (A). Granulosa cells were incubated with FSH (100 ng/ml) or TNF α (20 ng/ml) for different duration (0-60 min). Phospho-I κ B, total I κ B contents were measured by Western blot. Effects of over expression dominant negative I κ B on NF κ B binding activity induced by TNF α or FSH (B). Granulosa cells were transfected with pCMV or pCMV-I κ B-DN (4 μ g, 24h) prior to TNF α (20 ng/ml) or FSH (100 ng/ml) treatment. Nuclear proteins were extracted (30 min after TNF α or FSH) and NF κ B binding activity was assessed by EMSA. XIAP and total I κ B contents were measured by Western blot from whole cell lysate (24 h after TNF α and FSH). Representative images (top) and densitometric analysis (bottom) of XIAP protein contents and NF κ B-DNA binding activity (as fold of control) are shown. Mean $\pm$ SEM of three independent experiments. *, P < 0.05; **, P < 0.01 (compared with control); +, P < 0.05; ++, P < 0.01 (compared to cells transfected with pCMV-I κ B-DN and treated with TNF α).

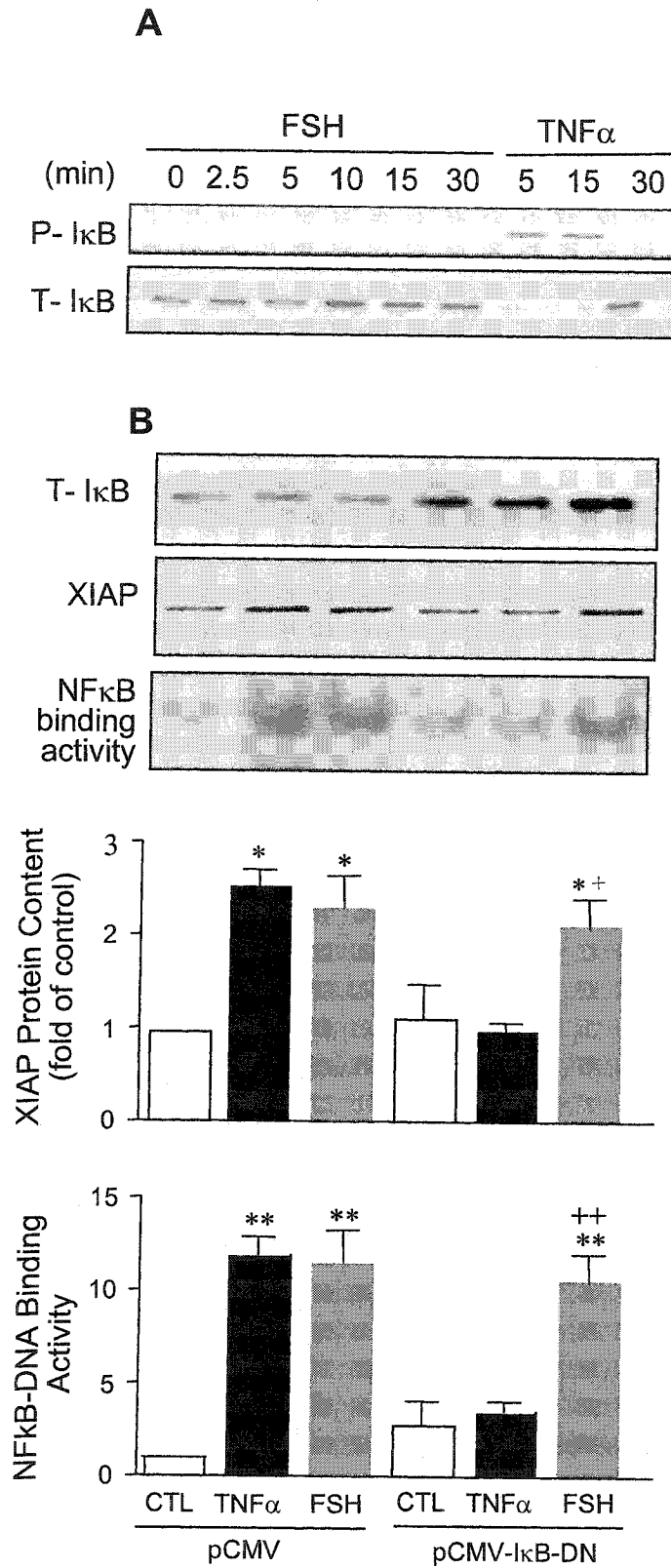


Fig 5

Fig 6 FSH increases phospho-Akt but not total Akt contents in granulosa cells *in vitro*. Granulosa cells were cultured for 30 min with different concentrations of FSH (0-150 ng/ml). Whole cell lysate were extracted (30 min after FSH) and Akt (total- and phospho-Akt) contents were measured by Western. Representative images (top) from three independent experiments and densitometric analysis (bottom) of Akt contents (defined as fold of control) are shown. Mean $\pm$ SEM of three independent experiments. *, $P < 0.05$ compared with control.

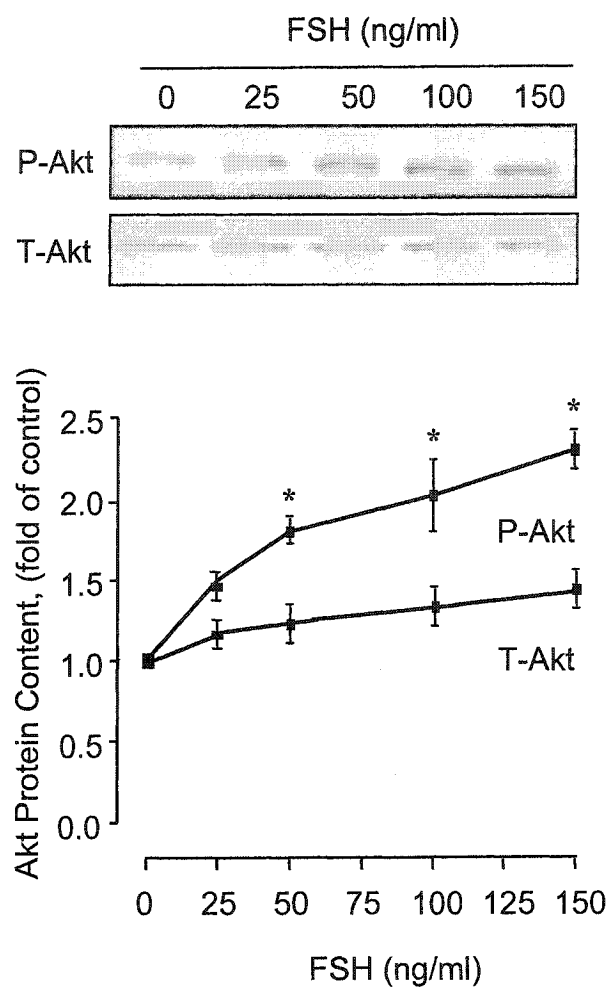


Fig 6

Fig 7 PI3K inhibitors (LY294002 and Wortmannin) suppress FSH-induced NFκB-DNA binding activity and XIAP contents in granulosa cells *in vitro*. Granulosa cells were cultured and pretreated (15 min) with different concentrations of LY294002 (0-20 :M) or Wortmannin (0-150 nM) prior to the addition of FSH (100 ng/ml). Nuclear proteins were extracted (30 min after FSH) and NFκB binding activity was assessed by EMSA. XIAP contents were measured by Western blot from whole cell lysate (24 h after FSH). Representative images (top) and densitometric analysis (bottom) of NFκB-DNA binding activity (defined as fold of control) are shown. Mean ± SEM of three independent experiments. *, P < 0.05; **, P < 0.01 compared with control.

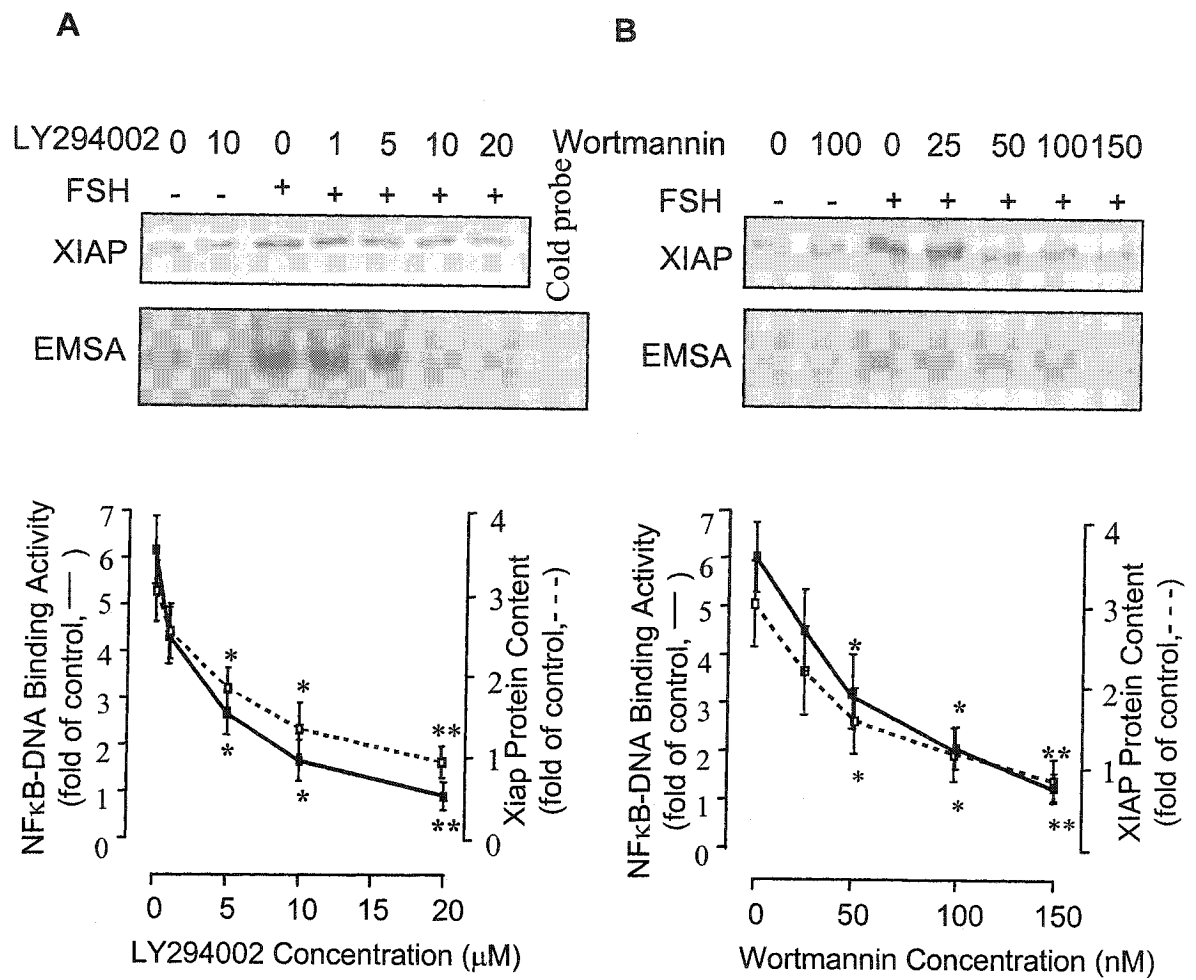


Fig 7

Preliminary studies from our laboratory have shown that FSH stimulates XIAP expression and the growth of rat ovarian follicles in culture (22). To assess if FSH-induced granulosa cell NF κ B activation is important in the gonadotropic regulation in follicular growth, pre-antral and early antral follicles were cultured with SN50 (100 ng/ml; SM50 as control) in the absence and presence of FSH (100 ng/ml). While follicles cultured in the absence of FSH but presence of SM50 showed a minimum growth in a 6-day culture period, addition of FSH significantly increased follicular volume ($P < 0.001$; Fig. 8). Pretreatment of follicles with SN50 before FSH addition resulted in a significant decrease in FSH-induced follicular growth ($P < 0.01$, FSH + SN50 *vs.* FSH + SM50 by repeated measurement two-way ANOVA; Fig. 8).

Fig 8 FSH-stimulated follicular growth is mediated through NF κ B activation. Follicles (160-210 μ m in diameter, 20 follicles per group) were cultured individually in 96 well plates and pretreated with SN50 or SM50 (100 ng/ml) and cultured in the absence or presence of FSH (100 ng/ml) for up to 6-day period. Follicular diameter was measured daily and changes in follicular volume are defined as the differences in volume between day n and day 0. The follicular volume at day-0 was 2.2 ± 0.25 nl, (n = 20). Mean $\pm$ SEM of three independent experiments. *, P < 0.05; **, P < 0.01 compared with control.

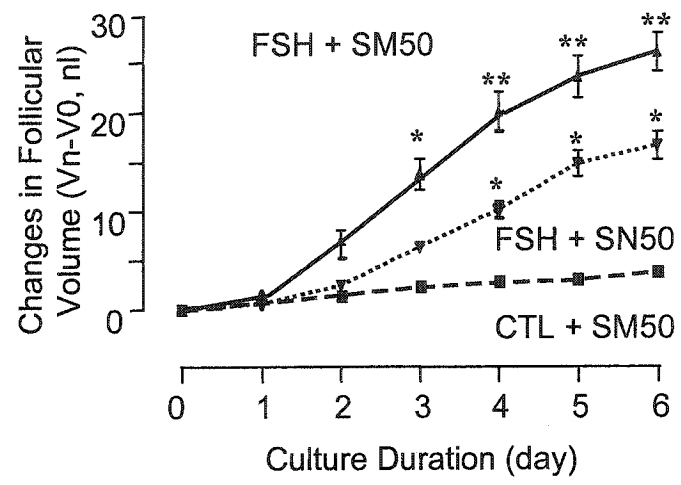


Fig 8

Discussion

Although the NF κ B family of transcription factors are important intracellular mediators of extra-cellular signals in a number of biological systems, whether they play a role in the gonadotropic regulation of ovarian follicular development is not known. The present study has demonstrated for the first time that FSH up-regulates XIAP expression and follicular growth through NF κ B activation. FSH induced p65-containing NF κ B translocation from cytoplasm to nucleus, and increased NF κ B-DNA binding activity shortly after gonadotropin challenge, a phenomenon that could be attenuated by SN50, a cell permeable inhibitory peptide of NF κ B translocation. Based on super shift EMSA results, the FSH-activated NF κ B contains p56 and p50 subunits. The gonadotropin-induced XIAP expression was also suppressed by SN50 (but not SM50, its inactive peptide), indicating that NF κ B is the mediator of this response. Interestingly, FSH also increased NF κ B-DNA binding activity in Sertoli cells, which exhibited higher basal levels of NF κ B-stimulated reporter gene expression. The activated NF κ B appeared to involve the p50 and p65 subunits and was specific to the stage of spermatogenesis (30). However, unlike the present studies, these investigations did not demonstrate the physiological function of FSH-activated NF κ B, particularly in the relation to regulation of a specific gene transcription in this testicular cell type.

There has been considerable progress in the current understanding of the signaling mechanism in NF κ B activation by cytokines, such as TNF α and interleukin (IL)-1 β (42). TNF α activates I κ B kinase (IKK), which is responsible for I κ B phosphorylation at serine 32 and 36. I κ B α is consequently ubiquitinated and degraded by 26S proteasome, resulting in the unmasking of the nuclear localization signal (NLS) of NF κ B and its

translocation to the nucleus. $\text{TNF}\alpha$ increases nuclear $\text{NF}\kappa\text{B}$ -DNA binding activity via this classical pathway. In contrast, FSH increases $\text{NF}\kappa\text{B}$ translocation and DNA binding without detectable changes in phospho-I κ B or total I κ B contents and degradation. Furthermore, over-expression of dominant negative I κ B (mutation at the phosphorylation sites) also failed to suppress FSH-induced $\text{NF}\kappa\text{B}$ activation and XIAP expression, while effectively attenuated the responses induced by $\text{TNF}\alpha$. These findings demonstrate, for the first time, that FSH activates $\text{NF}\kappa\text{B}$ through a pathway independent of I κ B phosphorylation and degradation.

In the present studies, we have demonstrated that FSH increases granulosa cell phospho-Akt content and $\text{NF}\kappa\text{B}$ -DNA binding activity, the latter response being readily suppressed by the presence of LY294002 or Wortmannin. Likewise, FSH-induced XIAP expression is sensitive to these PI 3K inhibitors. Taken together, these findings suggest that the $\text{NF}\kappa\text{B}$ activation and subsequent *XIAP* gene expression in response to FSH is PI3K-dependent. Recently, Reddy *et al.* also reported that the tumor suppressor PTEN, an endogenous antagonist of PI3K, inhibits IL-1 β -induced $\text{NF}\kappa\text{B}$ activation in glioma cells without interfering with the I κ B phosphorylation and degradation pathway (43). Furthermore, IL-1 increases phosphorylation of p65 and p50 subunits of $\text{NF}\kappa\text{B}$ and subsequent activation of $\text{NF}\kappa\text{B}$ pathway, which was blocked by LY294002 (44). These findings not only are consistent with our concept that FSH-induced XIAP expression is mediated through PI3K-dependent $\text{NF}\kappa\text{B}$ pathway, but also raise the possibility that the increase of granulosa cell PI3K activity in response to the gonadotropin, may involve $\text{NF}\kappa\text{B}$ phosphorylation. Whether $\text{NF}\kappa\text{B}$ phosphorylation is a result of the direct action of

an activated-Akt or secondary to the phosphorylation and activation of an as yet unknown downstream kinase(s) remains to be investigated.

Using a follicle culture system, we have previously demonstrated that FSH induces follicular XIAP expression, suppresses granulosa cell apoptosis and stimulates follicular growth *in vitro* (7). The present studies suggest that while FSH activates granulosa cell PI3K-dependent NF κ B pathway, addition of SN50 to follicle cultures attenuates FSH-induced follicular growth, supporting our hypothesis that FSH-induced follicular growth involves suppression of apoptosis mediated by a cell survival factor (*e.g.* XIAP) and NF κ B activation by PI3K. Consistent with this concept is the recent demonstration that PI3K pathway is involved in NF κ B-mediated Bcl-X_L gene expression during CD40 signaling (45). In contrast, evidence also exists in Theileria-transformed leucocytes that activation of the PI3K-Akt pathway is not directly linked to NF κ B (46). The PI3K-Akt pathway does not contribute to the persistent induction of I κ B α phosphorylation as well as NF κ B and transcriptional activation. In human endothelial cells, TNF α and IL-1 activate a PI3K/Akt pathway and that the anti-apoptotic effect of Akt is also independent of NF κ B (47). These results raise the possibility that the NF κ B-dependence of the PI3K cell survival pathway maybe agonist- and cell type- specific.

The role of cAMP in NF κ B activation in granulosa cells is not known. It is well established that FSH increases granulosa cell cAMP level and activates protein kinase A (PKA, (48)). PKA is known to directly phosphorylate NF κ B p65, promote its association with co-activators and consequently increase NF κ B transcriptional activity in LPS-challenged 70Z/3 cells (49;50). In addition, activation of NF κ B in HeLa and B cells by

TNF α is associated with phosphorylation of I κ B α , NF κ B precursors and p65 subunit and is modulated by the presence of shrimp alkaline phosphatase or potato acid phosphatase (51). Stimulation of Sertoli cells with activators of the cAMP-PKA signaling pathway (e.g. forskolin or FSH) also increases NF κ B–DNA binding activity. While preliminary data from our laboratory have shown that cAMP can mimic FSH in XIAP up-regulation in rat granulosa cells, if and how the cAMP/PKA pathway is involved in the FSH-induced, NF κ B-mediated XIAP expression in granulosa cells remains to be determined.

In conclusion, our findings support our hypothesis that the binding of FSH to granulosa cell receptor induces NF κ B activation and translocation to the cell nucleus and increases XIAP gene transcription. This process is mediated through PI3K/Akt pathway and is I κ B phosphorylation- and degradation-independent. However, whether the increase in XIAP mRNA observed in the present studies was indeed a consequence of increased gene expression and/or increased mRNA stability requires further experimentation. Moreover, whether and how the cAMP/PKA pathway is involved in this regulation, remains to be investigated.

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CHAPTER 5 UNPUBLISHED DATA

5.1 INDUCTION OF OVULATION *IN VITRO*

To assess the ability of cultured follicles to respond to an ovulatory concentration of hCG, follicle cultures (see Material and Methods, Chapter 2) were supplemented with hCG (5 IU/well/follicle, i.e. 0.5 IU/ml) at the end of a four-day culture period to induce ovulation *in vitro* (Fig. 1). Oocyte maturation [as evident by the presence of the first polar body upon the cumulus cell removal by trypsin treatment (0.1%, w/v, 1-3 min, 37°C and gentle shaking; Fig. 1, insert)] was assessed 18 h after the initiation of hCG treatment. Whereas hCG was ineffective in control follicles, it significantly increased ovulation rate in follicles maintained in the presence of FSH (100 ng/ml; 64%; $p < 0.001$; Table 1). Ovulation could also be induced in follicles with a delayed FSH exposure (i.e. FSH only present on Days 3 and 4) but was at a significantly lower rate (34%; $p < 0.05$; Table 1). These results suggest that FSH-primed pre- (160-180 μm) or early-antral (180-210 μm) follicles *in vitro* can produce matured oocytes upon hCG induction.

The reason why only 64% of cultured follicles response to ovulation induction is not clear. It is possible that follicles selected for the present studies have a large size range (160-210 μm) and are of preantral and early antral follicles. By the end of the 4-day culture period when hCG was added, the follicles were at different development stages and follicular responsiveness to gonadotropins. Thus, at the time when follicles were exposed to hCG, the follicular estrogen level as well as gonadotropic (FSH and/or LH) receptor abundance on the granulosa and theca cells are different, resulting in significant variation of cellular responsiveness to ovulatory hCG challenge. It is possible

Fig. 1 A representative image of ovulated follicle with extruded oocyte.

An ovulated follicle (FL) with oocyte surrounded by cumulus cells (OCC) after a four days of culture in the presence of FSH (100 ng/ml) and in response to hCG (50 IU/ml) *in vitro*. Insert shows an extruded oocyte (OC) with first polar body (FPB) following cumulus cell removal by trypsinization.

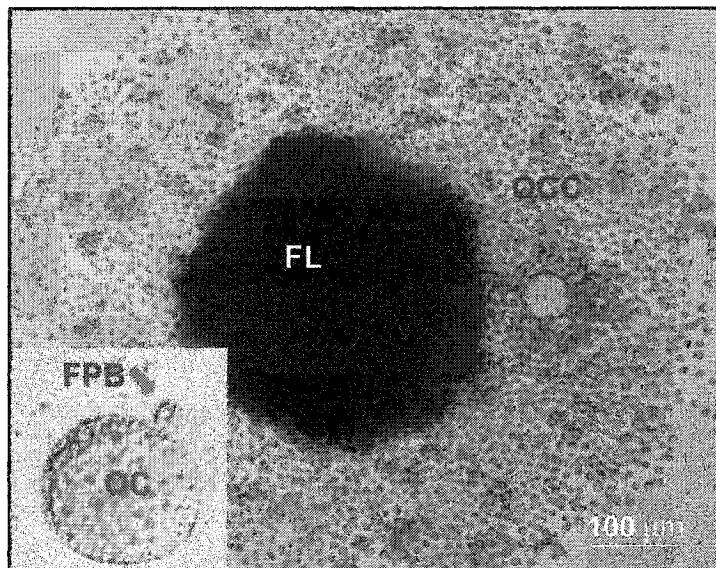


Fig 1

Table 1. Effect and Duration of FSH Exposure on Ovulation in Rat Follicle Culture

FSH exposure (100 ng/ ml)	No of follicles observed	Non-ovulated follicles	Ovulated follicles	Ovulation Rate (%)
No	20	20	0	0
Days 3-4*	28	18	10	34
Days 0-4 **, +	48	17	31	64

Chi-square analysis of differences in the number of ovulated verses non-ovulated follicles between treatment groups :

* $p < 0.05$ (Days 3-4 vs. No), ** $p < 0.01$ (Days 0-4 vs. No)

+ $P < 0.05$ (Days 0-4 vs. Days 3-4)

that if the culture period extended for another one or two days to allow further development of these earlier stage follicles and a higher ovulation rate could be expected.

5.2 LACK OF EFFECTS OF ESTROGEN IN GRANULOSA CELLS ON XIAP EXPRESSION *IN VITRO*

A number of studies have shown that regulation of apoptosis by steroid is tissue- or cell- specific, and that the same steroid may exert opposite actions in different tissues. Estrogens prevent apoptosis in ovarian cells (Billig et al 1993), while androgens prevent apoptosis in the ventral prostate (Kyprianou et al 1998) and progesterone inhibit apoptosis in the uterus but not in the ovary (Rocco et al 1992). In addition, glucocorticoids induce apoptosis in the thymus (Wyllie et al 1980; Compton and Cidlowski 1986) whereas cortisol treatment has no effect on ovarian DNA fragmentation. It will be of interest to determine whether these steroids regulate apoptosis through regulating IAP expression (Billig et al 1993).

The estrogen receptor (ER) is a ligand-activated enhancer protein, a member of the steroid/nuclear receptor superfamily. Upon ligand binding, ER undergoes conformational changes (activation), forming a ligand- ER complex and binds to estrogen response elements (EREs, 5'-GGTCAnnnTGACC-3'). Most estrogen-regulated genes contain imperfect, non-palindromic EREs. However, stimulation of target gene expression by 17 β -estradiol (E2) is thought to be mediated by two mechanisms: (i) 'direct binding' where the E2-ER complex binds directly to ERE and interacts with coactivators and components of the RNA polymerase II transcription initiation complex,

resulting in enhanced transcription; (ii) 'tethering' where ER interacts with another DNA-bound transcription factor in a way that stabilizes DNA binding of that transcription factor/or recruits coactivators to the complex. Although there is no ERE on *XIAP* gene, it is possible that the expression of XIAP may be up-regulated through interaction with yet undetermined transcription factor(s) via the latter mechanism described above. To determine if estrogens have a direct regulatory role on XIAP expression in the ovary, granulosa cells harvested from eCG-primed rat ovaries were cultured with estradiol-17 β (0, 0.08, 0.4, 2 μ g/ml) for 24 h (as described in Material and Methods, Chapter 4). XIAP contents were assessed by Western blotting. 17 β -estradiol alone had no significant effect on XIAP protein contents *in vitro* (Fig. 2). This is consistent with earlier contention (Chapter three) that estrogen indirectly regulates XIAP gene expression via another intra ovarian factor (e.g. theca-derived TGF α) through a paracrine pathway.

5.3 FSH UP-REGULATES GRANULOSA CELL RIAP EXPRESSION IN VITRO.

Although the present studies were focused on the role and regulation of ovarian XIAP expression, HIAP-2 has also been demonstrated to exist in ovary and to response to gonadotropin *in vivo* (Li et al 1998b). A dose-response study of FSH on rat HIAP-2 (RIAP) was performed. Granulosa cells from eCG-primed immature rat ovaries (see Material and Methods, Chapter 4) were cultured in the presence of FSH (0-100 ng/ml). RIAP protein content in whole cell lysate was assessed by Western blotting. FSH significantly increased RIAP level in a concentration-dependent manner (Fig. 3). The role of RIAP in the gonadotropic-stimulated ovarian follicular development remains to be determined.

Fig. 2 Western blot showing the lack of effects of estradiol on granulosa cell XIAP content *in vitro*.

Granulosa cells were cultured with different concentrations of estrogen (0-2 mg/ml) for 24 h. Whole cell lysate was extracted and XIAP content was assessed by Western blot. Densitometric XIAP value was normalized against total protein stained with Ponceau S (0.2%) to avoid loading difference. XIAP content was expressed as fold of control. Representative image (*top*) and densitometric analysis (*bottom*) of XIAP contents are shown. Values represent Mean $\pm$ SEM of three independent experiments. Data were Arcsine square root transformed before One Way ANOVA.

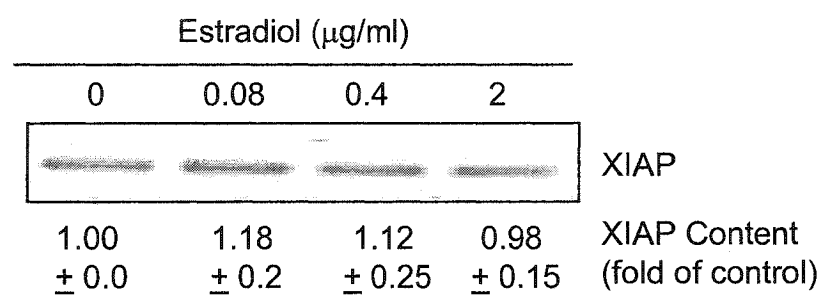


Fig 2

Fig. 3 FSH increases RIAP contents in rat granulosa cells.

Rat granulosa cells were cultured with different concentrations of FSH (0-150 ng/ml) for 24 h. Whole cell lysate was extracted and RIAP content was assessed by Western blot. Densitometric RIAP value was normalized against total protein stained with Ponceau S (0.2%) to avoid loading difference. RIAP content was expressed as fold of control. Representative image (*top*) and densitometric analysis (*bottom*) of RIAP contents (normalized against total protein and expressed as fold of control) are shown. Each value represents Mean $\pm$ SEM of three independent experiments. Data were Arcsine square root transformed before One Way ANOVA. Result indicates that FSH significantly up-regulates RIAP content. **, $p < 0.01$.

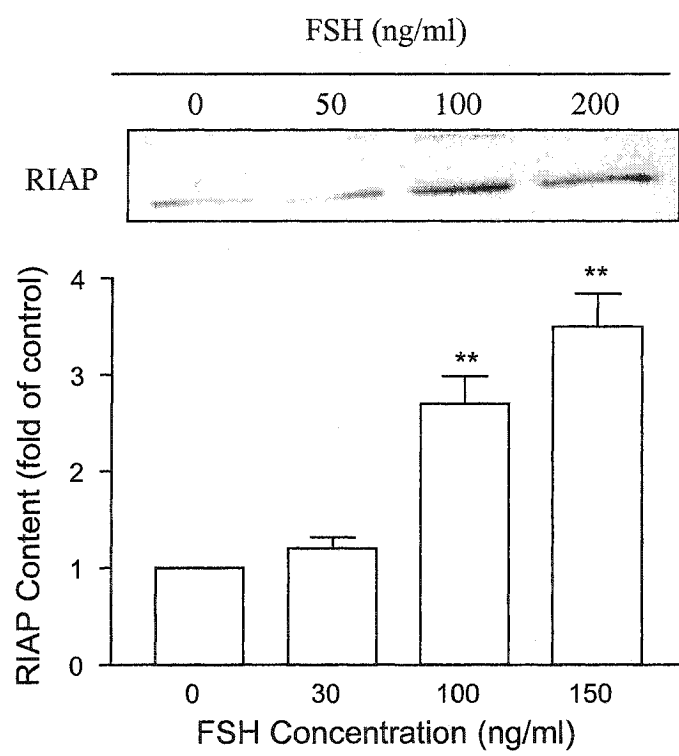


Fig 3

5.4 CYCLIC ADENOSINE MONOPHOSPHATE (cAMP) INCREASES XIAP PROTEIN LEVELS IN RAT GRANULOSA CELLS

Cyclic adenosine monophosphate is a well-established second messenger for FSH action in granulosa cells. By binding to its receptor on the granulosa cells, FSH stimulates the production of cAMP, which results in the activation of cyclic AMP-dependent protein kinase A (PKA). A preliminary study on the effect of db-cAMP on XIAP content was carried out. Granulosa cells from eCG-primed immature rats were cultured as described in Material and Methods of Chapter 4. Upon an overnight plating (18h) of 0.7 million-granulosa cells per well (6-well plate), cells reached 70% confluence. They were washed once with serum-free RPMI 1640 and treated with different concentrations of dibutyryl-cyclic AMP (db-cAMP, 0-400 μ M). Western blot analysis of granulosa cell extracts indicates that XIAP can be up regulated by cAMP (Fig. 4) with a significant response seen at 100 μ M and a greater response at 400 μ M. These results indicate that cAMP can mimic FSH-induced XIAP up-regulation, although mechanism through which this is achieved is yet to be investigated. Further studies are needed to define the role of the gonadotropic regulation of XIAP expression during ovarian follicular development and atresia.

5.5 ADDITIONAL INFORMATION ADDED SUBSEQUENT TO THESIS DEFENCE

Sensitivity of TGF α and estradiol ELISA (P123-124) were 0.3 pg/ml and 10 ng/ml, respectively.

Fig. 4 Dibutyl-cyclic AMP (db-cAMP) increases XIAP levels in rat granulosa cells *in vitro*.

Rat granulosa cells were cultured with different concentrations of db-cAMP (0-400 μ M) for 24 h. Whole cell lysate was extracted and XIAP content was assessed by Western blot. Densitometric XIAP level was normalized against total protein stained with Ponceau S (0.2%) to avoid loading difference. Representative image (*top*) and densitometric analysis (*bottom*) of XIAP contents (normalized against total protein and expressed as fold of control) are shown. Each value represents Mean $\pm$ SEM of three independent experiments. Data were Arcsine square root transformed before One Way ANOVA. Result indicates that db-cAMP significantly up-regulates XIAP content. *, $p < 0.05$; **, $p < 0.01$.

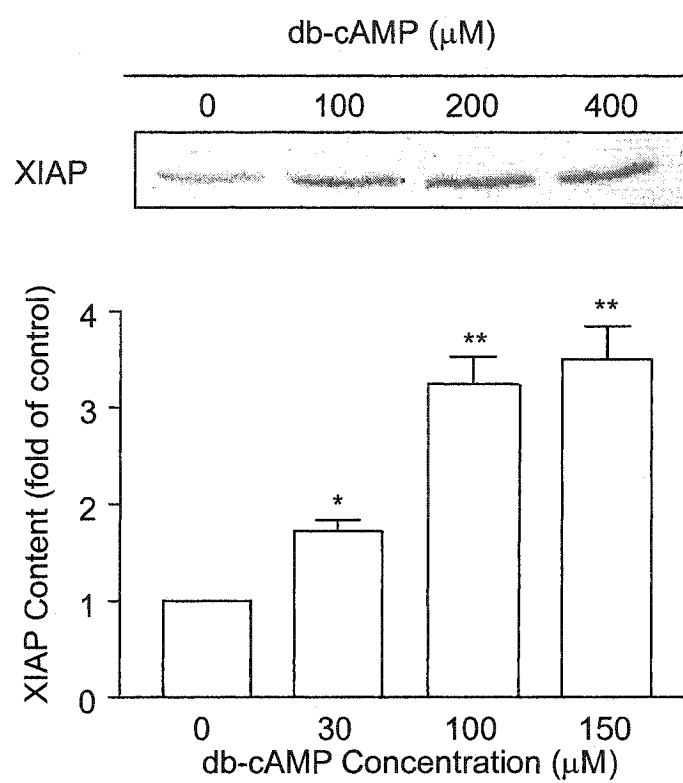


Fig 4

CHAPTER 6 GENERAL DISCUSSION

6.1 MAJOR FINDINGS AND THE SIGNIFICANCE OF THE THESIS PROJECT

FSH is an important survival factor and key determinant of dominant follicle selection during follicular development. However, further study is required on the cellular mechanisms and the signaling pathways by which FSH elicits its anti-apoptotic action. Previous studies have demonstrated that cell death (Fas/FasL) and survival (IAPs) genes are regulated by gonadotropic stimulation or withdrawal during follicular development and atresia (Kim et al 1998; Kim et al 1999; Li 1998b; Jose de los et al 2000; Asselin et al 2001b). IAP proteins are key intrinsic regulators of apoptosis (LaCasse et al 1998; Cheng et al 2002; Asselin et al 2000), which block cell death primarily by inhibiting distinct caspases. Although other proteins (e.g. FLIP) have been identified to inhibit upstream caspases, only the IAPs have been demonstrated to be endogenous repressors of the terminal caspases. In this context, IAPs set up an endogenous threshold level for caspase activation, thus determining the sensitivity of the cell to various apoptotic stimuli. Recent studies suggest that XIAP is involved in the regulation of a number of cellular functions (see Holcik et al 2001 for a review). XIAP is highly expressed in healthy but not in atretic follicles in the rat ovary (Li et al 1998b). In addition, the granulosa cell XIAP level is high in FSH-stimulated follicular development, while gonadotropin withdrawal decreases granulosa cell IAP content and induces apoptosis (Li et al 1998b). Gonadotropin-induced, XIAP-mediated apoptosis suppression has been suggested to play an important role of oocyte survival during folliculogenesis.

(Matsumoto et al 1999). However, the physiological role of XIAP in follicular development and atresia remains unclear and is the subject of this study.

Using a rat follicle culture system coupled with an adenoviral gene delivery procedure, I have examined in the present study, the role of FSH in the regulation of XIAP expression and the importance of XIAP in the gonadotropin-induced follicular development. It has been shown that FSH stimulates granulosa cell XIAP expression and follicular growth, as well as suppresses apoptosis (Wang et al 2003) (Chapter 2). Overexpression of XIAP potentiates the FSH-induced follicular cell proliferation and growth while XIAP down-regulation attenuates the actions of FSH. These results indicate that the changes in XIAP expression induced by FSH are critical for the maintenance of normal follicular growth and development. However, XIAP upregulation alone is insufficient for granulosa cell proliferation and follicular growth, suggesting that other intra-ovarian factors, such as TGF α are also involved in the stimulation of follicular growth. My studies have demonstrated that FSH-stimulated follicular development *in vitro* is mediated through an estrogen-dependent pathway involving the action of theca-derived TGF α (Wang et al 2002a), (Chapter 3). Up-regulation of XIAP, in response to FSH and TGF α , suppresses apoptosis in granulosa cells and facilitates follicular growth. This result is consistent with the theory that the interactions between theca and granulosa cells play an important role in FSH-induced follicular development (Tajima et al 2002b).

In addition to the second messengers calcium and cAMP in FSH signaling, the PI3K and NF κ B pathways appear to play an important role. I have shown, for the first time, that FSH induces NF κ B activation and translocation to the granulosa cell nucleus, a process involving the up-regulation of XIAP mRNA abundance and protein content

(Wang et al 2002b) (Chapter 4). This process appeared mediated through PI3K/Akt pathway but not the classical I κ B phosphorylation- and degradation pathway. These conclusions are supported by the following findings. FSH increased granulosa cell phospho-Akt content and NF κ B-DNA-binding activity, the latter response readily suppressed by the presence of LY294002 or wortmannin. Similarly, FSH-induced XIAP expression was sensitive to these PI3K inhibitors. However, the precise mechanism by which FSH activates PI3K/Akt and NF κ B pathways remains to be investigated. For example, Akt has been shown to be able to phosphorylate NF κ B (see Cheng et al 2002 for a review), whether it is a direct effect of Akt or mediated through other factors is yet to be studied. Also, whether the increase in XIAP mRNA observed in the present study was indeed a consequence of increased gene expression and / or mRNA stability requires further investigation.

6.2 INFORMATION GAP AND FUTURE DIRECTIONS

6.2.1 XIAP auto-ubiquitination/degradation and stability in gonadotropic-stimulated follicular development:

My current studies focused on gonadotropic regulation of XIAP mRNA abundance and protein content. However, the stability of XIAP is another important factor that influences net XIAP level. It has recently been demonstrated that several IAP proteins, including XIAP, are degraded in apoptotic thymocytes in an auto-ubiquitinated proteasome-dependent manner (Yang et al 2000). More importantly, XIAP has been found to possess ubiquitin ligase (E3) activity, suggesting that XIAP catalyzes its own

ubiquitination and that of other intra cellular proteins. The E3 ligase activity of XIAP is mediated by the RING Zn finger domain. Significantly, a RING-less XIAP version has been found to be degraded less effectively than the wild-type protein and this relative stability was correlated with enhanced cell survival (Yang et al 2000). Similarly, mutation within the RING domain that abolished Zn binding also eliminated E3 ligase activity (Yang et al 2000). HIAP-1 has been demonstrated, first in IAP family, can ubiquitinate caspases-3 and -7 but not caspase-1 in thymocyte lysates (Huang et al 2000). Recently, XIAP has been shown to target the active form of caspases-3 (Suzuki et al 2001b) and the IAP inhibitor Smac (MacFarlane et al 2002) for degradation by the 26 S proteasome. Thus, anti-apoptotic activity of these proteins may involve two distinct mechanisms: the enzymatic inhibition of caspase activity by BIR domains and specific targeting of caspases for proteasome-mediated degradation by its zinc ring finger motif. Whether these two events are complementary or mutually exclusive in the regulation of ovarian follicular development and atresia remains to be determined.

It is conceivable that either an enhancer or an inhibitor of XIAP auto-ubiquitination could be a regulatory candidate drug. The regulation of XIAP degradation and stability in gonadotropic-stimulated follicular development would be an interesting area to study. For example, the question, whether FSH-increased XIAP content is in part a consequence of inhibited XIAP degradation, needs to be addressed. In order to address this question, granulosa cells and follicles could be cultured in the absence or presence of FSH, and transfected with Zinc finger motif-deleted XIAP Myc-tagged cDNA. Ubiquitinated-XIAP contents can be determined by immuno-precipitation (using anti-Myc antibody) and subsequent Western blotting. If XIAP auto-ubiquitination and

degradation is the major mechanism by which XIAP is processed and is regulated by gonadotropin, we would expect significantly decreased ubiquitinated-XIAP contents in cells transfected with Zinc finger motif-deleted XIAP cDNA in the presence of FSH, than in control cells. It is also worthwhile to test if XIAP has a shorter half-life due to higher level of XIAP ubiquitination in atretic follicles than in healthy ones.

6.2.2 Regulators of XIAP function:

Our studies have shown that FSH is an important inducer of XIAP expression. As reviewed in the Introduction section, three negative regulators of XIAP have recently been identified, i.e. XAF1, DIABLO/Smac and HtrA2/Omi (Liston et al 2001; Srinivasula et al 2000; Du et al 2000; Verhagen et al 2000; Suzuki et al 2001a). These factors can block the inhibition of caspases by XIAP through directly binding to the anti-apoptotic factor (Liston et al 2001; Srinivasula et al 2000; Du et al 2000; Verhagen et al 2000; Suzuki et al 2001a). The regulation and influence of these regulators on XIAP function during ovarian follicular development is unknown and could be another area for future investigation.

The discovery of these three cellular proteins that interact with XIAP and modulate its anti-apoptotic activity is consistent with the critical role of XIAP in cellular homeostasis. It had been observed that over-expression of XIAP protects cells from different types of apoptotic triggers including UV-irradiation, γ -irradiation and chemotherapy drugs (Cheng et al 2002). The survival benefits of XIAP up-regulation in cells are obvious in the treatment of neuro-degenerative diseases. Conversely, the ability to modulate XIAP activity through a subset of cellular proteins would be essential for

maintaining tight control of the apoptotic process and preventing oncogenesis. XAF1 has been demonstrated as a tumor suppressor gene. XAF1 is expressed ubiquitously in all normal adult and fetal tissues but is present at very low or undetectable levels in a variety of cancer cell lines (Cheng et al 2002; Liston et al 2001). In contrast, XIAP levels were relatively high in the majority of cancer cell lines. Such high XIAP: XAF1 ratio in cancer cells may provide a survival advantage through the relative increase of XIAP anti-apoptotic function (Fong et al 2000). In addition, *in vitro* studies have demonstrated that XIAP suppresses caspase activation and cell death, and XAF1 antagonizes these activities. XIAP and NAIP have been demonstrated to be essential for intracellular signaling of glial cell-derived neurotrophic factor in motor neuron survival in neonatal rats (Perrelet et al 2002). On the other hand, it is possible that XAF1 may play a critical role in mediating the apoptotic induction of cancer cells by inhibiting the anti-apoptotic activity of XIAP (Liston et al 2001). It remains to be determined if a similar function exists for DIABLO/Smac and/or HtrA2/Omi. Possible roles of these negative regulators of XIAP in follicular development and atresia have not yet been reported. It is possible that XAF1 contents are low or nonfunctional in dominant follicles but high in atretic ones. Down-regulation or knockout of XAF1 and/or DIABLO/Smac may be a good approach to assess their role in the regulation of XIAP function and ovarian follicular growth.

6.2.2.1 Potential role of IRES in the regulation of XIAP translation:

As I have demonstrated, FSH increases XIAP granulosa cell mRNA abundance and protein content. The increase in FSH-induced XIAP mRNA level is mediated through NF κ B activation. The mechanism of post-transcriptional regulation on XIAP protein content remains unknown. XIAP is expressed as a 9 kb mRNA (Liston et al 1996). The coding region is only 1.5 kb long, leaving 5' and 3' untranslated region (UTRs) of 1.5 and 6 kb respectively. The presence of long 5' UTRs is rare in eukaryotic transcripts and it is believed that these long regions may interfere with efficient translation (Kozak 1989). Despite this unusual structure of XIAP mRNA, XIAP is expressed ubiquitously in most adult and fetal tissue (Liston et al 1996). Interestingly, it has been shown that a specific sequence, termed IRES (Internal Ribosome Entry Sequence), exists in the 5' UTR that facilitates efficient translation of XIAP mRNA (Holcik et al 1999). IRES-mediated translation has been first described for viral RNAs where it serves to initiate cap-independent translation of uncapped viral mRNA (Pelletier and Sonenberg 1988; Jang et al 1988). These elements were also discovered in a limited but growing number of cellular mRNAs (Holcik et al 2000a). Curiously, most of these mRNAs code for growth factors (VEGF, PDGF, IGF-II, FGF-2), oncogenes (c-myc, c-jun) and proteins involved in the control of apoptosis (Apaf-1, DAPS/p97/NAT1, XIAP). IRES-containing mRNAs are translated under several stress conditions including viral infection, growth factor deprivation, hypoxia and γ -irradiation-induced apoptosis, when most of the cellular protein synthesis is inhibited. It has been proposed that IRES-mediated translational control may have evolved to regulate cellular responses in acute but transient stress conditions (Holcik et al 2000a). The IRES-mediated translation of XIAP appears to be necessary for XIAP function and offers significantly greater

protection against apoptosis induced by serum-withdrawal than that is seen with a XIAP construct missing the IRES element (Holcik et al 1999). Furthermore, low dose γ -irradiation results in IRES-mediated translational up-regulation of XIAP that correlates with enhanced survival of some lung carcinoma cell lines, suggesting that IRES translation may in fact be critically involved in the progression of cancer (Holcik et al 2000b). It is well established that the LH surge is involved in the induction of ovulation, a process often associated with macrophage infiltration, cytokine release and increased vascular permeability. This is often considered an inflammatory response in the ovarian follicle. Previous studies have shown a rapid (within 3 h) increase in XIAP expression in cultured rat granulosa cells following $\text{TNF}\alpha$ treatment (Xiao et al 2001). Whether the acute XIAP up-regulation by $\text{TNF}\alpha$ is IRES-dependent is not known. Whether IRES-mediated XIAP expression plays an important role in follicular development and ovulation *in vivo* will be an interesting subject for future investigation.

6.2.3 Role of estrogen in granulosa cell XIAP expression

As shown in Chapter 5, estrogen alone had no effect on the changes of granulosa cell XIAP protein content. These results are consistent with the fact that there is no estrogen receptor-binding site on XIAP gene. However, as demonstrated in Chapter 3 (Wang et al 2002a), estrogen is a necessary mediator of FSH-induced $\text{TGF}\alpha$ secretion and the regulation of follicular growth, therefore indicating the interplay between endocrine (i.e. FSH) and paracrine (i.e. $\text{TGF}\alpha$ and estrogen) factors in the regulation of XIAP protein contents and ovarian follicular development.

6.3 ROLE OF XIAP AS A SIGNALING MOLECULE

XIAP appears to be a multifunctional protein involved in a number of diverse cellular processes. Recent studies have established that protection against apoptosis by XIAP is achieved by two separate mechanisms (Fig 1): 1) caspase related processes (e.g. caspase inhibition, caspase ubiquitination/degradation, modulating of caspase activation by ubiquitinating/degradation of its activator Smac, as well as XIAP auto-ubiquitination/degradation); 2) interaction with cell signaling pathways [e.g. up-regulation of the PI3K/Akt pathway, activation of in bone morphogenetic protein (BMP) and c-Jun N-terminal kinase (JNK) signaling]. Both mechanisms are BIR domain-dependent processes (Sanna et al 2002a).

6.3.1 Role of XIAP in PI3K/Akt pathway

XIAP has been demonstrated to play a role in the regulation of PI3K/Akt, while PIK/Akt pathway has been shown to play an important regulatory role in granulosa cell survival during follicular development. The activation of this survival pathway in granulosa cells is gonadotropic-mediated and follicular stage-dependent, and may involve an increase in phospho-Akt content (Asselin et al 2001b; Wang et al 2002b) (Chapter 4). Whereas gonadotropin increased granulosa cell XIAP and phospho-Akt contents, these responses were attenuated by gonadotropin withdrawal. Total Akt levels were not affected by these treatments (Asselin et al 2001b). XIAP over-expression increased the contents of phospho-Akt, but not of total Akt in both granulosa and ovarian cancer cells. In contrast, down-regulation of XIAP decreased phospho-Akt content and induced

Fig. 1 Modulation of cell signaling pathways by XIAP

Protection against apoptosis by XIAP is achieved by two separate mechanisms: caspase related processes and interaction with cell signaling pathways. XIAP inhibits both mitochondria and death receptor mediated apoptosis by inhibiting caspases and ubiquitinating caspase-3 and Smac, thereby facilitating their degradation. XIAP level can also be regulated by auto-ubiquitination. Activation of PI3K by growth factors leads to conversion of PI-4,5-P2 to PI-3,4,5-P3 (PIP3). PIP3 is required to recruit Akt, ILK, and PDK1 to the plasma membrane. Once these proteins have moved to the membrane, PDK1 and ILK phosphorylate and activate Akt. FSH up-regulates XIAP expression also through activation of the PI3K/Akt pathway (Chapter 4). In BMP mediated MAPK signaling pathway, XIAP binds to TGF- β receptors and TAB1. ILPIP is identified as a XIAP-interacting protein that significantly enhances this property of XIAP. This signaling occurs through TAB1/TAK1, which are necessary for activation the MAP kinase kinase, JNK1.

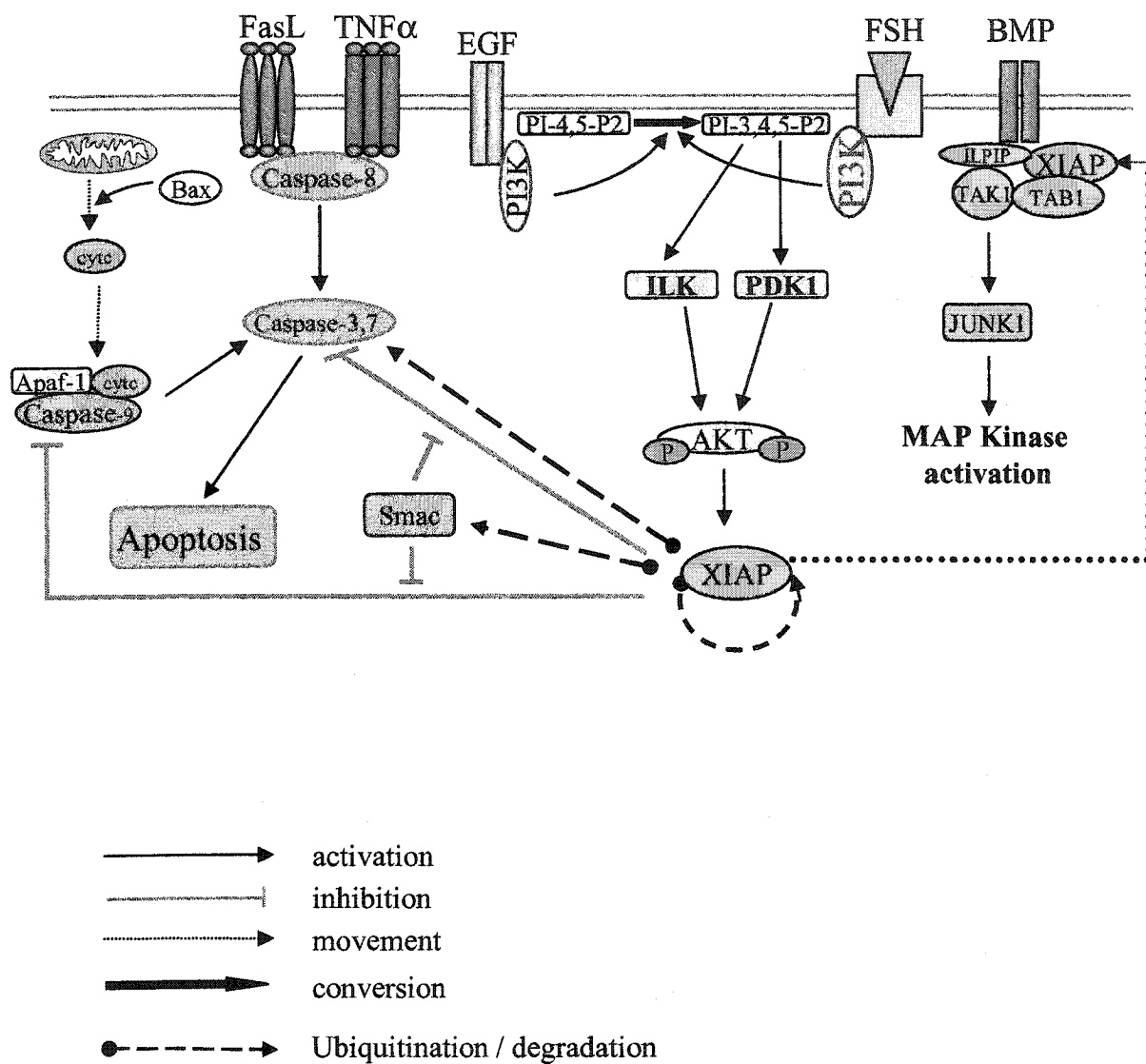


Fig 1

apoptosis (Asselin et al 2001a & 2001b). The relative abundance of XIAP may be a key element in cell fate determination, as it is a potent endogenous inhibitor of caspases. The precise mechanism(s) involved in the control of PI3K pathway by XIAP is not known. PI3K activation converts phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) and subsequently leads to Akt phosphorylation. It is possible that XIAP can influence PI3K directly or its downstream kinases including ILK and PDK1. Moreover, PIP3 can be reversed to PIP2 by phosphatases such as phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and Src homology 2 (SH2)-containing inositol phosphatase (SHIP). Despite the fact that both PTEN and SHIP have the ability to dephosphorylate a PI3K product, the role of PTEN, but not of SHIP, has been established in negatively regulating the PI3K/Akt cascade and in human myeloma leukemogenesis (Choi et al 2002). Alternatively, a recent study has demonstrated high ILK activity and Akt phosphorylation in PTEN-mutant prostate cancer cells and that PTEN wild type transfection suppressed ILK activity and Akt activation (Persad et al 2000). Since ILK is sensitive to and activated by high levels of PIP3 (Delcommenne et al 1998), mutation in the PTEN gene leads to PIP3 accumulation and ILK activation. However, which of these kinases and/or phosphatases are indeed putative candidates involved in the regulation of the phosphorylated (activated) status of Akt and survival in granulosa cells by XIAP remains to be determined.

6.3.2 Role of XIAP in BMP signaling

Ovarian follicular development involves regulation of gonadotropins and intra-ovarian factors through activation of multiple signaling pathways, such as these of BMP [via SMAD pathway, see Balemans and Van Hul 2002 for a review], TNF α (though NF κ B and caspases activation, see Beyaert et al 2002 for a review) and FSH (via cAMP, PI3K or Notch signaling pathways, see Richards et al 2002a for a review). Whether XIAP is involved in regulation of these pathways in the control of follicular development is not known.

Oocyte-granulosa cell communication involves the oocyte-derived factor BMP-15 and the granulosa cell-derived factor kit ligand, both of which have been shown to be crucial regulators of ovarian folliculogenesis (Otsuka and Shimasaki 2002). BMPs are members of the transforming growth factor β (TGF- β) family and are implicated in diverse cellular processes including cell growth, morphogenesis and differentiation (reviewed in Wotton and Massague 2001). BMP15 has been shown essential for female fertility and that natural mutations can cause both increased ovulation rate and infertility phenotypes in a dosage-sensitive manner (Galloway et al 2000). TAK1, a member of the MAP kinase family, and its activator, TAB1, have been shown to participate in BMP signaling pathway involved in mesoderm induction and patterning in early *Xenopus* embryos (Yamaguchi et al 1999). XIAP has been demonstrated as a TAB1-binding protein. XIAP associated not only with TAB1 but also with the BMP receptors, as a positive regulator linking the BMP in mammalian cells. The interaction of XIAP with TAB1 *in vitro* is mediated by the BIR domain region of XIAP (Yamaguchi et al 1999; Hawkins et al 2001; Sanna et al 2002a and 2002b). In contrast, the interaction of XIAP with the BMP receptor is facilitated by the RING Zn finger domain. It has been

demonstrated that XIAP is an activator of TAB1, which in turn activates the downstream MAP kinase family member TAK1 (Yamaguchi et al 1999). Although it is unclear how XIAP regulates BMP signaling upstream of TAB1-TAK1, it has been suggested that XIAP functions as an adapter protein that either stabilizes the TAB1-TAK1 complex or is directly involved in its recruitment to the receptor (Yamaguchi et al 1999). It is not known if the increased-granulosa cell XIAP content in response to FSH plays a role in BMP signaling during ovarian follicular development. Investigation into the cross-talk of the FSH and BMP signaling pathways would be of particular interest. These studies will provide new insight into the function of XIAP in the regulation of ovarian follicular growth.

6.3.2 Role of XIAP in JNK1 signaling

XIAP, but not HIAP-1 and HIAP-2, has been found to selectively activate JNK1, another downstream member of the MAP kinase-signaling pathway (Sanna et al 1998). This regulation appeared specific, as XIAP failed to activate JNK2, p38, ERK2, or ERK5/BMK1. In contrast to the activation of TAB1-TAK1, a BIR domain-dependent process, JNK1 was not found to physically associate with XIAP, suggesting that other proteins may be involved in the activation process. It is interesting to note that JNK1 is also rapidly activated following the stimulation of the BMP receptor (Massague et al 2000). While the precise mechanism of this activation is not known, it has been suggested that, at least in *Drosophila*, TAK1 could be involved in JNK1 activation (Takatsu et al 2000), and that binding of XIAP with both BMP receptor and TAB1 leads

to activation of TAK1 and, subsequently, of JNK1. XIAP-interacting protein (ILPIP) significantly enhances this property of XIAP. Interestingly, it is possible that JNK1 and XIAP are part of the same positive-feedback loop since protection of cells by XIAP in interleukin-1 β -converting enzyme-induced apoptosis is dependent on activated JNK1 (Sanna et al 1998). Sanna et al also showed that TAK1 was essential for the XIAP-mediated activation of JNK1 and protection from TNF- α and ICE-induced apoptosis (Sanna et al 2002a & 2002b). JNK cascade has been demonstrated to be essential for the normal morphogenesis of the dorsal appendages and the micropyle during oogenesis (Suzanne et al 2001). However, it is not known if the increase in XIAP expression in response to gonadotropin acts through JNK1 and promotes follicular survival and development. In the present study, I have shown the essential involvement of XIAP expression in the gonadotropic regulation of follicular growth. To test the role of JNK1 in this process, the influence of XIAP over-expression (adenoviral XIAP sense infection) on JNK1 and phosphorylated-JNK1 (activated JNK1) contents can be examined in follicles cultured in the presence or absence of FSH. In addition, if transfection of constitutively activated JNK1 is capable of reversing the suppression of follicular growth induced by XIAP down-regulation (as shown in Chapter 2), the role of JNK1 in the gonadotropin-stimulated XIAP-mediated follicular development would be confirmed.

6.4 ROLE OF OTHER IAPs IN OVARIAN FOLLICULAR DEVELOPMENT

Although this study is focused on XIAP, other IAP members have also been shown to play a role in the gonadotropin-stimulated follicular growth. It has been

demonstrated that HIAP-2 has high abundance in healthy follicles but not apoptotic follicles (Li et al 1998b). Like that of XIAP, Hiap-2 expression in both granulosa and theca cells increased throughout the follicular maturation, reaching maximal levels at the antral stage of development. Gonadotropin treatment increased Hiap-2 and XIAP protein content and suppressed apoptosis in granulosa cells, resulting in the development of follicles to the antral and preovulatory stages. Due to the important cell survival role of XIAP, XIAP knockout mice are expected to have lethal or severe abnormal developmental problems. However, a recent report of XIAP knockout mice having no phenotypic changes but with a significant high level of HIAP-2 suggests the existence of a compensatory mechanism possibly involving HIAP-2 (Harlin et al 2001).

The newly identified IAP member Survivin also exists in normal ovaries (Johnson et al 2002) and is found in high abundance in ovarian tumors (Yoshida et al 2001). Unlike HIAP-2 and XIAP, Survivin is mitogenic. The promoter of the mammalian *SURVIVIN* gene contains several elements typical of cell cycle-regulated genes and *SURVIVIN* is regulated in a cell cycle-dependent manner in normal cells (Li et al 1998a). Survivin mRNA and protein accumulate selectively during or around the time of mitosis (Reed and Bischoff 2000). Survivin lacks a long extension of the BIR domain that XIAP uses to access the active site of the caspase enzyme. Survivin may bind caspase-9, since its BIR domain is closely related in three-dimensional structure to the XIAP BIR3 domain, which binds and inhibits this enzyme *in vitro*. Phosphorylation of Survivin on threonine 34 (T34) is necessary for its association with processed caspase-9. To date, several models have been proposed to explain how Survivin inhibits caspases: 1) directly binds and inhibits caspase, as XIAP; 2) suppresses Smac expression, thus enhance the

inhibition of caspases by IAPs; and 3) enhances the function of IAPs, a function opposite to Smac (Reed 2001). Although there are numerous studies on Survivin in carcinomas, the function of Survivin in gonadotropin-induced follicular development and especially the cross talks between Survivin and XIAP, has not been investigated.

6.5 ROLE OF CYCLIC ADENOSINE MONOPHOSPHATE (cAMP) IN THE REGULATION OF GRANULOSA CELL XIAP EXPRESSION DURING FOLLICULAR DEVELOPMENT.

Although cAMP is a well-established second messenger of FSH, it is not known whether cAMP has a role in gonadotropic regulation of XIAP expression. Our preliminary experiments indicate that exogenous dibutyl cAMP can mimic FSH-induced XIAP upregulation, although the mechanism involved is not known. The present studies (Chapter 4) have also demonstrated a gonadotropic-increased p-Akt in granulosa cells. Although Akt is not an FSH-inducible kinase, it is constitutively expressed in granulosa cells. It has been demonstrated that Akt is phosphorylated as a consequence of FSH stimulation of cAMP (Richards et al 2002a; Khan et al 2002; Gonzalez-Robayna et al 2000). While the majority of PKA remains in the cytoplasm, a fraction is translocated into the nucleus where it phosphorylates key nuclear transcription proteins, most notably cAMP response element binding protein (CREB). Although we have been unable to locate the classical CREB binding site on the XIAP gene, recent studies suggest that this binding site is not necessary for CREB regulated gene expression (Roesler 2000). FSH can up-regulate activated CREB levels (Shell et al 2002). As CREB is known to be

activated by certain cell survival pathways (i.e. NF κ B and IGF-1) (Gu et al 2002; Walton and Dragunow 2000), it is possible that CREB is another mediator involved in XIAP up-regulation by FSH. cAMP-CREB pathway regulates the survival, and possibly the differentiation and function, of newborn neurons.

In neuronal cells, AKT has been found to induce CREB activation (Walton and Dragunow 2000). NF κ B can increase CREB expression in Sertoli cells (Delfino and Walker 1999). NF κ B may affect activated CREB level by increasing the quantity of CREB available for phosphorylation by AKT (Fig.2). It is also possible that the PI3K/NF κ B and cAMP pathways act in a synergistic manner. If PKA activates kinases in the PI3K/NF κ B pathway, NF κ B and AKT both act to increase activated CREB (downstream of cAMP), it would appear that the two pathways could amplify their effects on XIAP expression. There is no evidence to support this speculation, thus more research is necessary before role of the two pathways in FSH-induced XIAP up-regulation can be completely understood. The study may help clarify the mechanism with which granulosa cell fate, and thus follicle destiny, is determined.

6.6 OVARIAN FOLLICULAR CULTURE SYSTEMS

In vitro ovarian follicle culture systems have been employed for study of normal ovarian physiology, treatment of infertility and germ line preservation (Smitz and Cortvrindt 2002). It has become a useful tool for studying follicle development and offers the potential to preserve reproductive options in cases of polycystic ovarian

Fig. 2 A hypothetical model illustrating possible interactions between the PI3K/NF- κ B signaling pathway and the cAMP signaling pathway for FSH in granulosa cells.

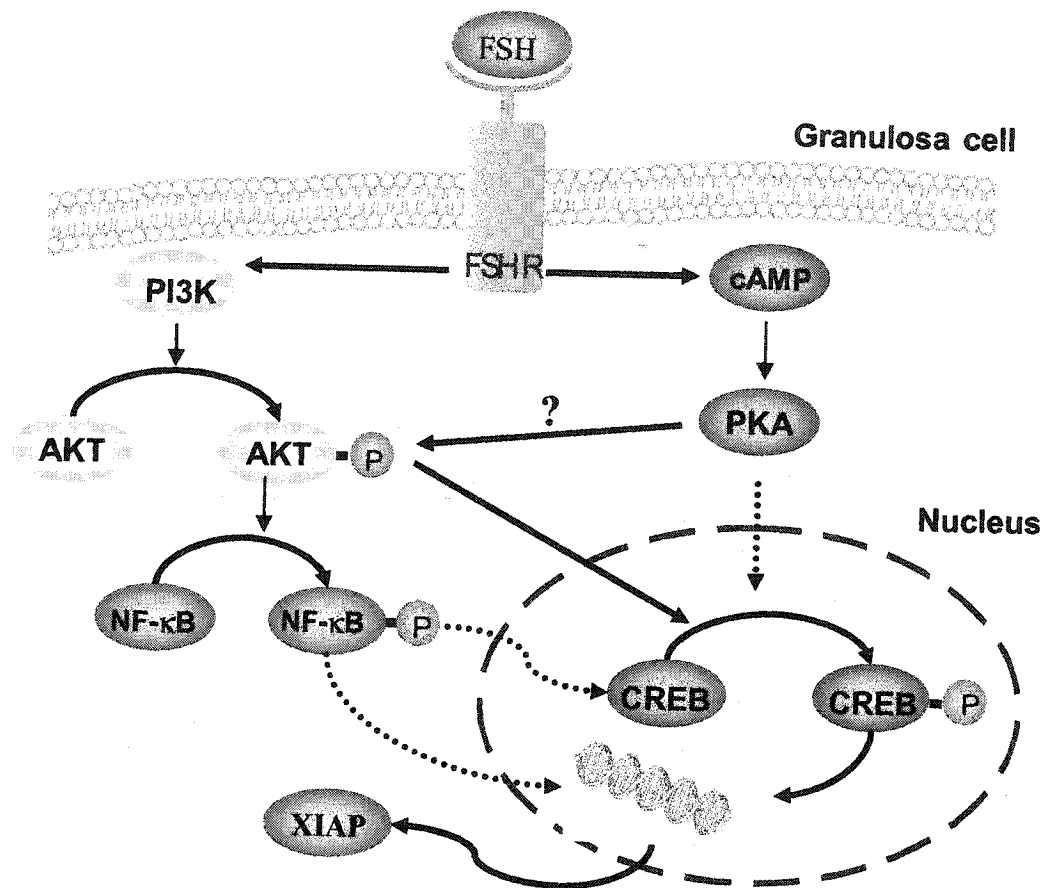


Fig 2

syndrome (PCOS), premature ovarian failure, or definitive sterility (post-oncotherapy). This culture system can also be used to examine factors that regulate follicle development and may ultimately provide treatment for reproductive infertility. In recent years several follicle culture systems have been established in different mammalian species for studying ovarian folliculogenesis and culturing immature oocytes (see Hartshorne 1997; Smitz and Cortvrindt 2002 for a review). Follicular culture systems can be used to study the different stages of follicles and their physiological function as well as their interactions.

Results from studies on the possible influence of gonadotropins and cytokines on early ovarian follicular genesis have been contradictory (see Kezele et al 2002 for a review). Primordial follicle cultures (Eppig and O'Brien 1996; Shaw and Trounson 2002; Picton 2001) allow investigators to study the influence of endocrine or paracrine factors (Devine et al 2002) as well as environmental toxicants (Smitz and Cortvrindt 2002) on early stage of folliculogenesis. Moreover, a major aim of the development of *in vitro* ovarian follicle culture systems in reproductive medicine is to develop fertilizable oocytes from immature female gametes of early follicle stages. Since *de novo* formation of female gamete stops by the time of birth, primordial follicles are the most abundant stage of follicles in ovary throughout the reproductive lifetime in mammals. Thus, theoretically cryo-preservation and subsequent culture of primordial follicles should be the most attractive strategy in reproductive medicine. To date, the mouse is the only model in which a live offspring has been produced from cultured follicles. Although frozen-thawed ovarian tissue grafts are useful in restoring fertility in a number of oophorectomized species, isolated primordial follicles remains arrested at the primary

follicle stage during *in vitro* development. To meet their requirements for growth, metabolism and differentiation, a triple-stage process involving ovarian explants cultures, isolation of granulosa-oocyte complexes and completion of meiotic maturation, has been developed (Gosden et al 2002; see Cortvrindt and Smitz 2002 for a review).

Culture of early antral follicles, follicles at a stage in which dominance is conferred, is another important and attractive approach to study follicular development in *vitro*. As reported in the present studies, pre- and early-antral follicles cultured for six days in the presence of FSH reached preovulatory stage (Wang et al. 2002a & 2002b; Wang et al 2003; Zhao et al 2000; McGee 2000; Rose et al 1999). Moreover, the present study has demonstrated for the first time that manipulation of a gene of interest in the smallest functional unit of ovary (follicle) can effect phenotypic changes in follicular development. In future, this system could be extended for the assessment of the influence of proteins or drugs *in vitro* on ovulation, oocyte maturation, fertilization and pregnancy outcome.

While the studies discussed above are concerned with cultures of individual follicles, cultures of multiple follicles has also been established and used to study the interactions between follicles of different stages as reported by Liu et al (Liu et al 2000). This culture system allows one to examine the role of dominant follicle and its regulation of subordinate follicles during ovarian follicular development.

6.7 CONCLUSION

Apoptosis is an important mechanism for the regulation of cell death during ovarian follicular atresia. However our knowledge of the intracellular regulation of follicular destiny is limited and a variety of unknown factors may be involved in this complex process. The multiple biological activities of XIAP, together with its unique translational and post-translational control make it a promising molecular target for modulating apoptosis and possibly treatment of apoptosis-associated ovarian diseases. One possible promising strategy is the over-expression or delivery of XIAP dominant negatives (e.g. XAF1, DIABLO/Sma and HtrA2/Omi) to inhibit XIAP function in target cells. Cell-specific gene targeting and delivery of the candidate “molecule” is the key determinant of the success of these therapeutic approaches. The outcome of these developments will have profound effects on management of patients with drug-resistant ovarian cancer and / or ovarian dysfunction. However, whether the FSH-increased granulosa cell XIAP expression, as observed in the current studies, was indeed a consequence of increased gene expression and/or increased mRNA/protein stability requires further experimentation. In addition, the generation of a potent and specific XIAP and Akt inhibitors would be an asset to studies on processes mediated by XIAP and Akt in the same way as inhibitors of MAP kinase 1 (e.g. PD98059, PD184352, U0126) have increased on our understanding of processes regulated by the classical MAP kinase pathway. Investigations of relative expression of death inducers and survival factors as well as the cross-talk between their signaling pathways will not only increase our knowledge on the physiological basis of ovarian follicular development and atresia, but

also provide important clues to better understand the pathobiology of ovarian disorders, including polycystic ovarian disease, premature ovarian failure and luteal phase defect.

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CHAPTER 8 BIBLIOGRAPHY

EDUCATION

- 9/98-present Ph.D. in Physiology Program, Department of Cellular and Molecular Medicine, University of Ottawa, Ontario, Canada
- 3/94 - 2/96 Department of Physiology (Requirements for M. Sc. program completed), College of Medicine, Nanjing University, Nanjing, People's Republic of China.
- 7/82 - 7/86 B.Sc., Department of Biochemistry, Nanjing University, Nanjing, People's Republic of China.

OTHER TRAINING

- 03/96 – 06/98 Visiting scholar, Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA.

ACADEMIC MEMBERSHIP AND INVITED TALKS

- 06/2000-present Member, Society for the Study of Reproduction
- 08/89-present Member, Society of Chinese Medicine
- 01/12/2000 “*Cloning of Mammalian Embryos*”, Presentation at Obstetrics and Gynecology Grand Rounds, University of Ottawa.
- 05/12/2000 Presentation of 13 lectures on *Reproductive Technologies* to senior high school students, as a part of the *Let's Talk Science Program*.

SCHOLARSHIPS, AWARDS AND SPECIAL RECOGNITION

- | | | |
|---|--|------------|
| • Lalor Foundation Travel Award | XIV Ovarian Workshop, Serono Symposia | 2002 |
| • Trainee Research Award Finalist | 33 rd annual meeting of Society of the Study for Reproduction | 2000 |
| • The Gerry Taichman Award | Department of Cellular & Molecular Medicine, Ottawa University | 2000 |
| • NSERC Scholarship | | 1999-2001 |
| • Excellence Scholarship | University of Ottawa | 1998-2001 |
| • Ontario Graduate Scholarship for Science and Technology | | 1998-1999 |
| • Admission Scholarship | University of Ottawa | 1998-1999. |

- Science and Technology Progress Award, 2nd place, People Liberation Army, People's Republic of China 1995
- Science and Technology Progress Award, 4th place, People Liberation Army, People's Republic of China 1994
- Undergraduate Student Award of Excellence Nanjing University, People's Republic of China 1986

PUBLICATIONS

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8. **Wang, Y.**, Liu, C.J., Cheng, H.L. The cryopreservation of rat and human pituitary tissue and cells. *Chinese Journal of Experimental Surgery Suppl*: 148, 1996.

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10. Shi, J.X., **Wang, Y.**, Liu, C.J. The influence of sialic acid levels in the cultured pituitary cells on their immunogenicity. *Journal of Chinese Medicine* 16: 111-117, 1994.
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12. **Wang, Y.**, Shi, J.X., Liu, W.X., Shao, X.G., Cheng, H.W., Liu, C.J. An experimental study of pituitary transplantation in rabbits. *Chinese Journal of Medicine* 105: 415-419, 1992. (In English)
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2. Jiang JY., Cheung CKM., **Wang Y.**, Tsang BK. Regulation of cell death and cell survival gene expression during ovarian follicular development and atresia. *Frontiers in Bioscience* 8:d222-237, 2003.

MANUSCRIPT SUBMITTED OR IN PREPARATION:

1. **Wang, Y.F.**, Meyer, K.B., Schmidt, K., Wan, S., Degen, S.J.F., and LaBarbera, A.R. Cloning, sequencing and expression of a new porcine follicle stimulating hormone receptor (pFSHR) cDNA. Molecular Reproduction and Development. (Submitted)
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PRESENTATIONS AT NATIONAL AND INTERNATIONAL MEETINGS (Outside People's Republic of China)

1. **Wang Y.**, Chan S. and Tsang B.K., Involvement of inhibitory nuclear factor- κ B (NF κ B)-independent NF κ B activation in the gonadotrophic regulation of X-linked inhibitor of apoptosis expression during ovarian follicular development *in vitro*. XIV Ovarian Workshop, Serono Symposia USA, Inc. July 27th-31st, 2002, Baltimore, Maryland, USA.
2. **Wang Y.**, Chan S. and Tsang B.K., Role of NF κ B in gonadotrophic regulation of X-linked inhibitor of apoptosis protein (XIAP) expression in rat ovarian granulosa cell *in vitro*. The 34th Annual Meeting of Society of the Study for Reproduction, July 25th-28th, 2001, Ottawa, Ontario, Canada.
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