

**REGULATION OF APOPTOSIS AND  
STEROIDOGENESIS  
IN  
OVARIAN PREEVULATORY GRANULOSA CELLS**

**EVA SVENSSON**

Department of Physiology and Pharmacology  
Göteborg University

**GÖTEBORG  
SWEDEN**

## ABSTRACT

Only a minute fraction of ovarian follicles present in a foetal ovary will complete the path to ovulation. The majority of the follicles will instead undergo atresia, a hormonally controlled apoptotic process. Apoptosis occurs throughout follicle development, with an extensive reduction in the number of follicles present at birth. Interestingly, this drastic reduction is not present among preovulatory follicles responding to the ovulatory surge of luteinizing hormone (LH), since the number of corpora lutea roughly equals the number of preovulatory follicles. Granulosa cells (surrounding the oocyte in the follicle) exposed to the LH-surge, are characterized by the induction of expression of progesterone receptors (PR) as well as a prominent steroidogenic activity. It was therefore pertinent to study factors involved in PR signalling as well as in regulation of steroidogenesis, to try to elucidate mechanisms regulating ovarian granulosa cell apoptosis.

Human as well as rat granulosa cells were isolated after *in vivo* treatment with hormones to induce luteinization of ovarian follicles. To study apoptosis, internucleosomal DNA fragmentation and caspase-3 activity, two characteristic apoptotic events, were used as endpoints. Cell concentration was shown to be an important factor to take into consideration when interpreting *in vitro* data, since a cell-concentration-dependent apoptosis was demonstrated in cultured granulosa cells. Internucleosomal DNA fragmentation was shown to be decreased in granulosa cells isolated from rats treated with human chorionic gonadotropin to mimic the endogenous LH-surge. Exposure to the LH-surge is a prerequisite for PR expression in granulosa cells to occur. To be able to interfere with PR signalling, two different PR-antagonists, RU 486 and Org 31710 were used. Addition of PR-antagonists to granulosa cell cultures during 24 h incubation in serum-free medium caused a dose-dependent increase in internucleosomal DNA fragmentation as well as an increase in caspase-3 activity compared to controls. These effects were not present in granulosa cells not exposed to the LH-surge *in vivo*, consequently not expressing functional PR. LH receptor stimulation of granulosa cells thus makes the granulosa cells less susceptible to apoptosis. This event seems to be at least partly, mediated by PR expression and activation.

Leptin is an adipocyte-derived hormone known to affect fertility by interfering with the central regulation of gonadotropin secretion. In addition, direct effects on ovarian cells may be postulated, regulating events in ovarian granulosa cells. Using the reverse transcriptase-polymerase chain reaction, we were able to detect the presence of leptin receptors in the human ovary but no expression of leptin mRNA. However, leptin was detected in follicular fluid, suggesting a possible endocrine action of leptin in the ovary. Stimulation of primary cultures of human luteinizing granulosa cells with leptin suppressed LH-induced estradiol production, indicating a mechanism for leptin to regulate fertility at the ovarian level. Whether this interferes with regulation of granulosa cell apoptosis remains to be determined.

In conclusion, this thesis provides insight into important mechanisms involved in regulation of ovarian luteinizing granulosa cell apoptosis, namely signalling via the progesterone receptor and interference with the granulosa cell steroidogenic capacity by leptin.

**Key words:** apoptosis, granulosa cells, luteinizing hormone, progesterone receptor, RU 486, Org 31710, leptin, steroidogenesis

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## LIST OF PUBLICATIONS

This thesis is based upon the following papers, which will be referred to in the text by their Roman numerals:

- I Svensson E Ch, Utter M, Leandersson P, Thelander H and Billig H. Cell-concentration-dependent apoptosis in cultured rat granulosa cells.  
*Submitted.*
  
- II Svensson E Ch, Markström E, Andersson M and Billig H.  
Progesterone receptor-mediated inhibition of apoptosis in granulosa cells isolated from rats treated with human chorionic gonadotropin.  
*Biology of Reproduction, 2000; 63: 1457-1464.*
  
- III Svensson E Ch, Shao R, Markström E, Andersson M and Billig H.  
Progesterone receptor antagonists induce apoptosis in cultured human luteinizing granulosa cells: increase in caspase activity and DNA fragmentation.  
*Manuscript.*
  
- IV Karlsson C, Lindell K, Svensson E, Bergh C, Lind P, Billig H, Carlsson LMS and Carlsson B.  
Expression of functional leptin receptors in the human ovary.  
*Journal of Clinical Endocrinology and Metabolism, 1997; 82: 4144-4148.*

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## LIST OF ABBREVIATIONS

<b>3<math>\beta</math>-HSD</b>	3 $\beta$ -hydroxysteroid-dehydrogenase
<b>aa</b>	amino acids
<b>ACTH</b>	adrenocorticotrophic hormone
<b>ADAMTS-1</b>	a disintegrin and metalloproteinase with thrombospondin-like motifs
<b>AF</b>	activation function
<b>AIDS</b>	acquired immuno deficiency syndrome
<b>AIF</b>	apoptosis inducing factor
<b>ANOVA</b>	analysis of variance
<b>AP-1</b>	activator protein 1
<b>Bad</b>	Bcl-X <sub>L</sub> /Bcl-2 associated death promoter
<b>Bcl-2</b>	B-cell lymphoma/leukemia-2
<b>BMI</b>	body mass index
<b>bp</b>	base pair
<b>Boo</b>	Bcl-2 homologue of ovary
<b>BOD</b>	Bcl-2-related ovarian death gene
<b>BSA</b>	bovine serum albumin
<b>CAD</b>	caspase activated DNase
<b>cAMP</b>	cyclic adenosine-3',5'-monophosphate
<b>CARD</b>	caspase activation and recruitment domain
<b>cDNA</b>	complementary deoxyribonucleic acid
<b>CL</b>	corpus luteum
<b>cRNA</b>	complementary ribonucleic acid
<b>CSF</b>	cerebrospinal fluid
<b>DBD</b>	DNA binding domain
<b>DED</b>	death effector domain
<b>DISC</b>	death inducing signalling complex
<b>DHT</b>	dihydrotestosterone
<b>DIABLO</b>	direct IAP binding protein with low PI
<b>DNA</b>	deoxyribonucleic acid
<b>eCG</b>	equine chorionic gonadotropin
<b>ET</b>	embryo transfer
<b>FSH</b>	follicle stimulating hormone
<b>FSH-R</b>	follicle stimulating hormone receptor
<b>GABA</b>	gamma aminobutyric acid
<b>GnRH</b>	gonadotropin releasing hormone
<b>h</b>	human
<b>hCG</b>	human chorionic gonadotropin
<b>hER-<math>\alpha</math></b>	human estrogen receptor- $\alpha$
<b>IAP</b>	inhibitor of apoptosis protein
<b>ICAD</b>	inhibitor of caspase-activated DNase
<b>IGFBP-5</b>	insulin growth factor binding protein-5
<b>IGF-I</b>	insulin growth factor-I
<b>IRS-2</b>	insulin receptor substrate-2
<b>IVF</b>	in vitro fertilization
<b>JAK</b>	janus kinase

<b>LBD</b>	ligand binding domain
<b>LH</b>	luteinizing hormone
<b>LH-R</b>	luteinizing hormone receptor
<b>MAPK</b>	mitogen-activated protein kinase
<b>MMP</b>	matrix metalloproteinase
<b>Mn-Zn-SOD</b>	manganese-zinc-superoxide dismutase
<b>mRNA</b>	messenger ribonucleic acid
<b>NPY</b>	neuropeptide Y
<b>P450scc</b>	cholesterol desmolase
<b>PCO</b>	polycystic ovary syndrome
<b>PGS-2</b>	prostaglandin endoperoxide synthase-2
<b>PMSG</b>	pregnant mare's serum gonadotropin
<b>PR</b>	progesterone receptor
<b>PR-A</b>	progesterone receptor type A
<b>PRAKO</b>	progesterone receptor type A knockout
<b>PR-B</b>	progesterone receptor type B
<b>PRE</b>	progesterone responsive element
<b>PRKO</b>	progesterone receptor knockout
<b>rFSH</b>	recombinant follicle stimulating hormone
<b>ROS</b>	reactive oxygen species
<b>RPA</b>	RNase protection assay
<b>RT-PCR</b>	reverse transcriptase-polymerase chain reaction
<b>Smac</b>	second mitochondria-derived activator of caspases
<b>SOD</b>	superoxide dismutase
<b>SRB1</b>	scavenger receptor class B type I
<b>STAT</b>	signal transducers and activators of transcription





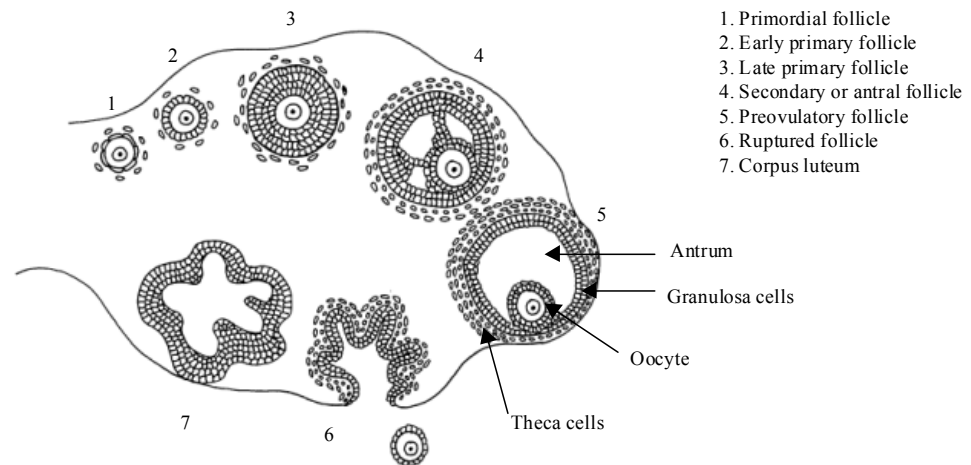
# INTRODUCTION

## FOLLICULAR DEVELOPMENT

Harbouring genetic information to be passed down from generation to generation, the ovaries are crucial structures for the survival of the species. The structural organization of the ovary is basically made up of two types of cells, germ cells and somatic cells. The germinal component originates from primordial germ cells that, after migration from the yolk sac, start to colonize the gonadal primordium during the fifth week post fertilization. The precursors of the somatic cells are found in the developing gonad (coelomic epithelium and mesenchymal cells) and in the neighbouring tissues (mesonephros). Together, these cells form the pool of resting primordial follicles in the ovary (Motta *et al.* 1997).

The foetal human ovary contains millions of germ cells, with a peak number observed at the fifth month of pregnancy ( $6.8 \times 10^6$  germ cells) (Baker 1963). However, up until birth, the number of germ cells decreases dramatically, the number of germ cells enclosed in primordial follicles at birth being less than 20% of its peak number (Baker 1963). There is solid evidence that oocyte attrition occurs through a programmed form of cell death called apoptosis (Reynaud and Driancourt 2000). Even after birth, the store of primordial follicles decreases with time, finally ending in the menopause as the consequence of the exhaustion of the pool of primordial follicles.

The maturation of ovarian follicles involves several sequential stages; initiation of growth of primordial follicles in the resting pool, growth of the follicles, selection of a dominant follicle, ovulation and finally luteinization.



**Figure 1.** A figure of an ovary showing the different developmental stages of a follicle.

Although the factor(s) initiating the growth and atresia of resting primordial follicles has (have) not yet been fully elucidated (Fortune *et al.* 2000, Gougeon 1996), the key endocrine events controlling follicular growth in mammals have been known for many years. The development of ovarian follicles comprises proliferation as well as differentiation of the somatic cells. During initiation of growth, the oocyte also enlarges (Gougeon 1996). Once resting primordial follicles have started to grow, basal concentrations of the gonadotropins, FSH (follicle stimulating hormone) and LH (luteinizing hormone), maintain growth up until the small follicular stage. FSH binds exclusively to FSH receptors (FSH-R) in granulosa cells,

whereas LH binds to LH receptors (LH-R), initially only localized to theca interna cells and then also to granulosa cells during maturation of the preovulatory follicle (Yamamoto *et al.* 1992). FSH-R have been detected on granulosa cells of human follicles from the secondary stage onward (Yamamoto *et al.* 1992). Receptors for LH appear in preovulatory granulosa cells, starting in the midfollicular phase (Shima *et al.* 1987, Yamamoto *et al.* 1992). Nonovulatory follicles in humans do not possess LH-R on their granulosa cells.

Interestingly, once an antrum has appeared within a follicle, there is a clear shift in the respective sensitivities to cell death of germ cells and somatic cells (Reynaud and Driancourt 2000). This coincides with the disappearance of DNase I, associated with programmed cell death/apoptosis, in the oocyte (Boone and Tsang 1997). Hence, the final tuning of the large number of growing follicles to a single ovulatory follicle is primarily achieved by somatic cell death (Hsueh *et al.* 1994, Reynaud and Driancourt 2000).

LH-mediated differentiation of theca cells involves an increased amount of mRNA for the LH-R as well as increased binding activity of the LH-R and an increased ability of thecal cells to synthesize aromatizable androgens. This increase is associated with the induction of mRNA and protein expression for 17- $\alpha$ -hydroxylase cytochrome P450, the key enzyme in converting progesterone to androstenedione (Richards 1994).

## **SELECTION OF THE FOLLICLE DESTINED FOR OVULATION**

Once selected, the growing dominant follicle acquires specific functional characteristics that permit it to differentiate to the preovulatory stage. One of the most important events is the appearance of aromatase in the granulosa cells.

Granulosa cells in small antral follicles start to express FSH receptors and are selected to continue growth by increases in basal concentrations of gonadotropins. From this stage the follicles become dependent on FSH for survival (Gougeon 1996). FSH-stimulation causes expression of aromatase in the granulosa cells, thus enabling the granulosa cells to convert androgens produced in the theca cells to estrogens. Circulating estrogen causes an inhibition of gonadotropin release. However, the selected follicle has been shown to have an aromatase activity much more sensitive to FSH than small follicles and is thus “favoured” in the situation of decreasing FSH-levels and still capable of producing large amounts of estrogens (Gougeon 1996).

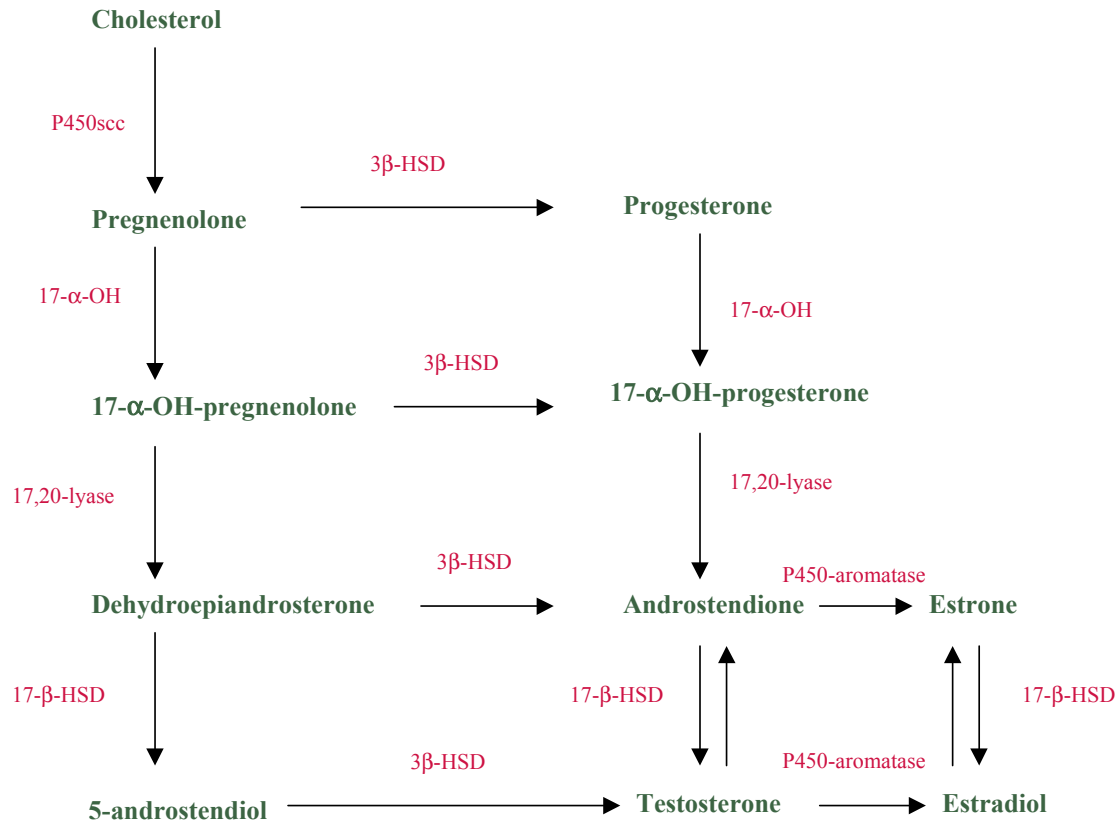
Interestingly, immunohistochemical studies have revealed estrogen receptor expression in granulosa cells only in the aromatase-positive antral or pre-ovulatory follicle (Suzuki *et al.* 1994). Aromatase expression appeared to occur only in a selected antral follicle in the folliculogenesis of the human ovary, suggesting that estradiol, locally synthesized by the granulosa cells in the selected follicle, may only act on these cells in the same follicle. In addition, it has been shown that during the second half of the follicular phase, the preovulatory follicle is much more vascularized than are less developed follicles (Gougeon 1996). Thus, this may be another mechanism, in addition to an “up-regulated” sensitivity of FSH, for the selected follicle to protect itself from the fall in plasma FSH concentrations. When a certain threshold of estrogen in plasma is reached, the negative feedback on gonadotropins is changed to a positive feedback, resulting in peak levels of LH, triggering ovulation and luteinization.

After the LH-peak, the granulosa cells become highly differentiated and stop proliferating, but instead produce high levels of progesterone. P450<sub>scc</sub> is the rate-limiting enzyme in converting cholesterol to pregnenolone. P450<sub>scc</sub> is negligible in granulosa cells of small follicles, but is increased in granulosa cells of preovulatory follicles. P450<sub>scc</sub> is also

expressed in thecal cells and interstitial cells. The conversion of pregnenolone to progesterone requires the enzyme 3 $\beta$ -HSD. This enzyme has been localized to the interstitial cells, theca cells and granulosa cells in large follicles and corpora lutea (Richards 1994).

The corpus luteum (CL) is a metabolically highly active structure with a high production rate of progesterone (Rothchild 1981). The CL in the non-pregnant woman actively synthesizes progesterone for 10-12 days.

### THE STEROID SYNTHESIS



P450scc: cholesterol desmolase

17- $\alpha$ -OH: 17- $\alpha$ -hydroxylase

3 $\beta$ -HSD: 3- $\beta$ -hydroxysteroid-dehydrogenase

17- $\beta$ -HSD: 17- $\beta$ -hydroxysteroid-dehydrogenase

*Figure 2. Schematic illustration of the steroid synthesis.*

## **ATRESIA**

Only a minute fraction of ovarian follicles present at birth complete the path to ovulation. The majority of follicles instead undergo atresia, which has been shown to be mediated by a highly organized type of cell death called apoptosis or programmed cell death (Billig *et al.* 1996, Hsueh *et al.* 1994, Kaipia and Hsueh 1997).

## **Apoptosis**

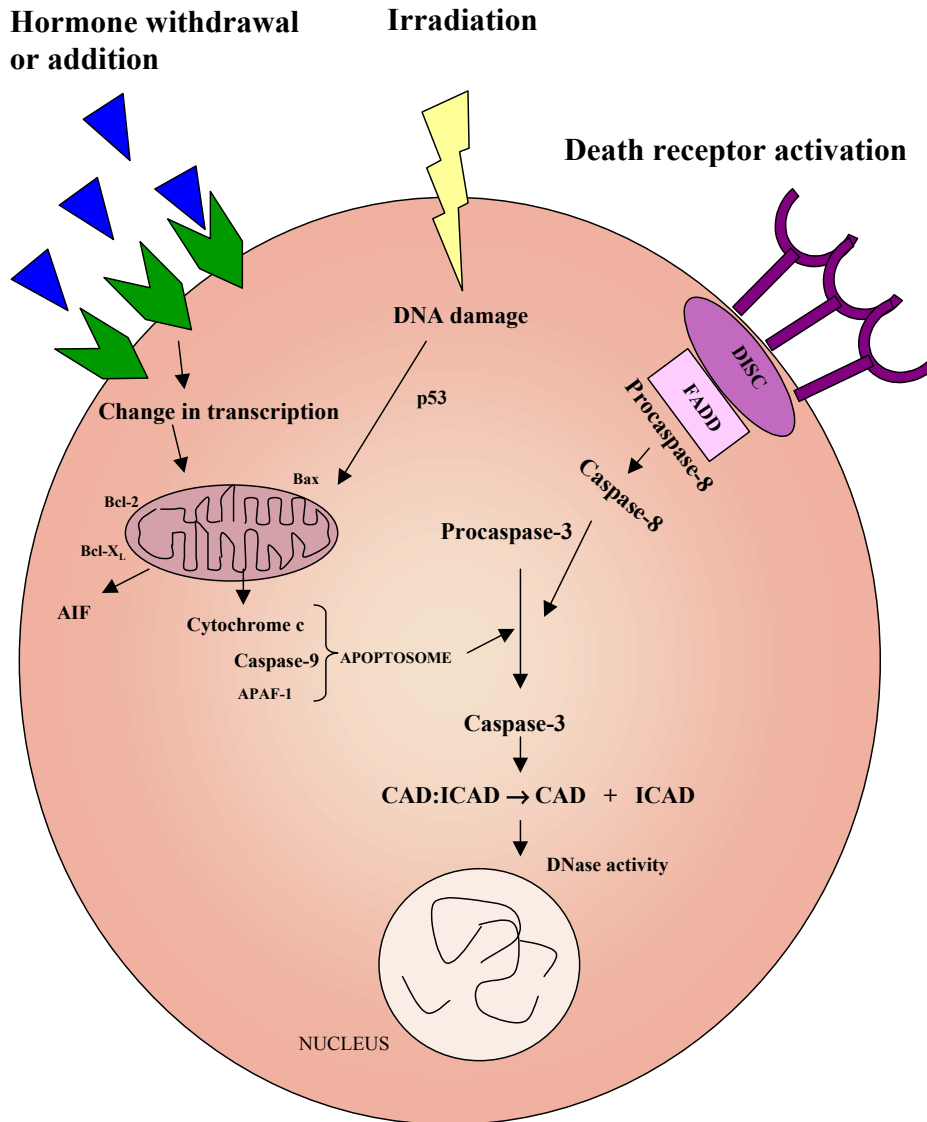
In 1972, Currie and colleagues coined the term apoptosis to describe a common type of cell death that they had repeatedly observed in various tissues and cell types (Kerr *et al.* 1972). The word originates from Greek and has the meaning of "falling off", like the petals from a flower or the leaves from a tree. Indeed, the general features of this kind of cell death had previously been described by several authors but had been designated several different names (Vaux and Korsmeyer 1999).

Apoptosis is an essential part of life for multicellular organisms. During foetal development, apoptosis is involved in organogenesis and it is important throughout life in the regulation of homeostasis in various tissues (Prindull 1995). Apoptosis in a physiological context, is tightly regulated and balanced against mitosis. Failure of this delicate regulation results in pathological conditions such as developmental defects, autoimmune diseases, neurodegeneration and cancer.

Characteristic apoptotic features include membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA fragmentation, finally ending in the engulfment of neighbouring cells, thereby avoiding an inflammatory response in surrounding tissues (Kaipia and Hsueh 1997, Savill and Fadok 2000). These changes are in bright contrast to morphological characteristics of necrosis, when the cell swells with subsequent rupture of the plasma membrane, with detrimental effects for surrounding cells (Falcieri *et al.* 1994).

A wide array of external signals may trigger the apoptotic machinery within a cell. These signals include irradiation-induced DNA damage, hormone addition or withdrawal and stimulation of certain "death receptors" (Billig *et al.* 1993, Billig *et al.* 1994, Hengartner 2000). Two major apoptotic pathways are depicted in Figure 3, namely the death receptor pathway and the mitochondrial pathway.

# APOPTOTIC PATHWAYS



**Figure 3.**

Oversimplified figure, showing two major apoptotic pathways in mammalian cells (modified after Hengartner 2000). **The death receptor pathway** is triggered by members of the death receptor superfamily (e.g. CD95, tumour necrosis factor receptor I). Binding of ligand to these receptors, causes receptor clustering and formation of a death inducing signalling complex (DISC). This complex recruits, via the adaptor molecule FADD (Fas-associated death domain protein), multiple procaspase-8 molecules, resulting in caspase-8 activation through induced proximity. **The mitochondrial pathway** is used extensively in response to extracellular apoptotic stimuli and internal insults such as DNA damage. These diverse response pathways converge on mitochondria, often through the activation of a pro-apoptotic member of the Bcl-2 family. Pro- and anti-apoptotic Bcl-2 family members meet at the surface of mitochondria, where they compete to regulate cytochrome c exit. If the pro-apoptotic side wins, an array of molecules is released from the mitochondrial compartment. Principal among these, is cytochrome c, which associates with Apaf-1 and then procaspase-9 to form the apoptosome. The death receptor and mitochondrial pathways converge at the level of caspase-3 activation. Downstream of caspase-3, the apoptotic programme branches into a multitude of subprogrammes, among which internucleosomal DNA fragmentation is one.

The apoptotic machinery comprises an intricate network of intracellular mediators. Two families of important regulators are the caspase family and the Bcl-2 family.

## **The caspase family**

Caspases are proteins highly conserved through evolution. Today over a dozen caspases have been identified in humans and about two-thirds of these have been suggested to function in apoptosis. Most of the visible changes that characterize apoptosis are brought about by caspases, why they are thought of as the central executioners of the apoptotic pathway (Hengartner 2000). All known caspases possess an active-site cysteine, and cleave substrates at Asp-Xxx bonds (that is after aspartic residues). Today, around 100 caspase substrates are known. The distinct substrate specificity is determined by the four residues amino-terminal to the cleavage site (Hengartner 2000).

One of the most used hallmarks of apoptosis is internucleosomal DNA fragmentation, brought about by an endonuclease, generating DNA fragments with lengths corresponding to multiple integers of approximately 180 basepairs (Wyllie 1980). The responsible endonuclease (called CAD for Caspase Activated DNase) pre-exists in living cells as an inactive complex with an inhibitory subunit, called ICAD (Inhibitor of Caspase-Activated DNase). Activation of CAD occurs by caspase-3-mediated cleavage of the inhibitory subunit, resulting in the release and activation of the catalytic subunit. Caspase-mediated cleavage of specific substrates also explains several other characteristic features of apoptosis, like cleavage of the nuclear lamins, required for nuclear shrinking and budding and cleavage of cytoskeletal proteins such as fodrin and gelsolin, causing loss of overall cell shape (Hengartner 2000).

Caspases themselves are synthesized as enzymatically inert zymogens, composed of three domains; an N-terminal prodomain and the p20 and p10 domains. There are three general mechanisms of caspase activation (Hengartner 2000);

### Processing by an upstream caspase

Most caspases are activated by proteolytic cleavage at two sites in the zymogen. Interestingly, all these cleavage sites occur at Asp-X sites, thus suggesting the possibility of autocatalytic activation (Thornberry *et al.* 1997). This "caspase-cascade" mode of caspase activation is used extensively by cells for the activation of the three downstream effector caspases, caspase-3, -6 and -7. These caspases are usually more abundant and active than the upstream "initiator" caspases e.g. caspase-8 and caspase-9. However, the caspase-cascade strategy for zymogen activation is not applicable to upstream caspase activation. Two other possibilities exist for activation of such caspases:

### Induced proximity

Caspase-8 is the key initiator caspase in the apoptotic "death receptor" pathway. Upon ligand binding, death receptors such as CD95 (also called Apo-1/Fas) aggregate and form membrane-bound signalling complexes. These complexes then recruit, through adapter proteins, several molecules of procaspase-8, resulting in a high local concentration of zymogen. The induced proximity model posits that under these crowded conditions, the low intrinsic protease activity of procaspase-8 is sufficient to allow the various proenzyme molecules to mutually cleave and activate each other.

### Association with a regulatory subunit

Unlike other caspases, proteolytic processing of procaspase-9 has only a minor effect on the enzyme's catalytic activity. Instead, association with a protein cofactor, Apaf-1, is required for caspase-9 activity. Together with cytochrome c, caspase-9 and Apaf-1 forms a complex called an apoptosome.

The communication between the upstream caspases and their regulators is mediated via protein-protein interaction modules, located in the prodomain of these caspases. Caspase-8 and -10 contain a death-effector domain (DED), whereas caspase-2 and -9 contain a caspase activation and recruitment domain (CARD). A third protein interaction module, present in several upstream regulators of apoptosis, such as CD95, is the death domain (Hengartner 2000).

### **The Bcl-2 family**

Another important set of apoptotic regulators is the Bcl-2 family. This family comprises anti-apoptotic members, such as Bcl-2 and Bcl-X<sub>L</sub>, as well as pro-apoptotic members, including Bax, Bid, Bik, BOD, Bok and Bcl-X<sub>S</sub> (Antonsson and Martinou 2000, Hsu *et al.* 1997, Hsu *et al.* 1998). The members of the Bcl-2 family can both homodimerize and heterodimerize, thereby regulating each other's activity. The key function of Bcl-2 family members seems to be to regulate the release of pro-apoptotic factors, in particular cytochrome c, from the mitochondrion into the cytosol (Antonsson and Martinou 2000).

### **The mitochondrion**

The mitochondrion is a crucial structure in the apoptotic machinery, harbouring several pro-apoptotic proteins, released to the cytoplasm upon a certain signal. One of these factors is cytochrome-c, one of the factors (in addition to Apaf-1) required for activation of caspase-9 in the cytosol (Li *et al.* 1997).

The question how cytochrome c manages to cross the mitochondrial outer membrane is not yet known, but the members of the Bcl-2 family are closely involved in the regulation of of this process. Three different models have been suggested for the Bcl-2-family regulation of cytochrome c release (Hengartner 2000);

1. Bcl-2 members form channels that facilitate protein transport. However, its unclear whether such channels would be big enough for proteins to pass through.
2. Bcl-2 members interact with other proteins to form channels. One candidate protein is the voltage dependent anion channel (VDAC).
3. Bcl-2 members induce rupture of the outer mitochondrial membrane.

Cytochrome c exit is an almost universal feature of apoptotic cell death. However, in some cases, it is a very late event, like in apoptosis induced by death receptors, where the mitochondrial pathway is often bypassed, and cytochrome c is released as a result of caspase activation (Scaffidi *et al.* 1998).

In addition to cytochrome c, other pro-death factors are stored in mitochondria, and will be released upon induction of apoptosis. Some examples are AIF (apoptosis inducing factor), Smac/DIABLO (second mitochondria-derived activator of caspases/ direct IAP-binding protein with low PI) and several procaspases, including procaspase-2, -3 and -9 (Hengartner 2000).

## The End

The last steps of apoptosis include rounding up of the cell and detachment from surrounding cells, membrane blebbing, packaging of cell content into apoptotic bodies and phagocytosis (Falcieri *et al.* 1994, Savill and Fadok 2000). The recognition of an apoptotic cell by neighbouring cells and macrophages has been intensively studied. Apoptotic cells show up a "come and eat me-flag" in due time. One of these flags are phosphatidylserine, which is normally present on the cytoplasmic side of the cell membrane, but which flips over to the external face of the cell membrane when a cell undergoes apoptosis. This event triggers the phagocytosis reaction (Savill and Fadok 2000). Recently, scavenger receptor class B type I (SRBI), expressed in theca cells and recognizing phosphatidylserine, was shown to recognize apoptotic granulosa cells (Svensson *et al.* 1999).

## Regulation of apoptosis in the ovary

Gonadotropins are required for the growth and development of ovarian follicles (Chun *et al.* 1994). If preovulatory gonadotropin surges are blocked or serum gonadotropins are decreased following hypophysectomy, follicles undergo atresia (Braw and Tsafiriri 1980, Braw *et al.* 1981). However, studies using cultured rat granulosa cells have shown that treatments with FSH, LH/hCG or insulin growth factor (IGF-I) are ineffective in the prevention of spontaneous apoptosis, despite their apoptosis-suppressing action in cultured rat follicles (Billig *et al.* 1996). This indicates the importance of neighbouring theca cells and local factors produced in the ovary for regulation of follicle growth and atresia.

Other follicle survival factors, e.g. epidermal growth factor/transforming growth factor, basic fibroblast growth factor (Tilly *et al.* 1992), interleukin-1 $\beta$  (Chun *et al.* 1995) and growth hormone (Eisenhauer *et al.* 1995) as well as pro-apoptotic factors, e.g. tumour necrosis factor- $\alpha$  (Kaipia *et al.* 1996), Fas ligand (Quirk *et al.* 1995) and GnRH (Billig *et al.* 1994) have been characterized. The action of IGF-I is locally modulated by IGF-binding proteins (IGFBPs), counteracting the protective effect of IGF-I on follicle apoptosis (e.g. IGFBP-3) (Chun *et al.* 1994). Activin inhibits granulosa cells apoptosis in a dose-dependent manner and the expression of activin subunits increases with the size of the follicle (Chun *et al.* 1996). Other important regulators of follicle survival and atresia are sex steroids. In the rat ovary, estrogens act as survival factors, whereas androgens promote apoptosis (Billig *et al.* 1993).

The dependence on transcription and translation for ovarian apoptosis was recently demonstrated by Svanberg and Billig (Svanberg and Billig 1999).

Many of the members of the Bcl-2 family have been isolated in the ovary. The anti-apoptotic Boo (Bcl-2 homologue of ovary) expression is highly restricted to the ovary and the epididymis (Song *et al.* 1999) and Bok (Bcl-2 related ovarian killer) is highly expressed in the ovary, testis and uterus (Hsu *et al.* 1997). Other members of the Bcl-2 family which have been isolated in the ovary are Bad (Bcl-X<sub>L</sub>/Bcl-2 associated death promoter), BOD (Bcl-2-related ovarian death gene) (Hsu *et al.* 1998), Bax, Bcl-2 and Bcl-X<sub>L</sub> (Tilly *et al.* 1995).

Although the major site of apoptotic DNA fragmentation in the ovarian follicle is the granulosa cell layer, theca cell apoptosis occurs, but rather late in the apoptotic demise of the follicle. The mechanism behind apoptosis in theca cells involves the Bcl-2 family and the caspase family (Foghi *et al.* 1998).



## Oxidative stress as an inducer of apoptosis

In addition to receptor-mediated regulation of apoptosis, the *in vitro* condition itself may be crucial to cell survival. Cell-plating density has been shown to be an important factor influencing the development of apoptosis in different culture systems, including chondrocytes, lens epithelial cells and hepatocytes (Bruckner *et al.* 1989, Ishizaki *et al.* 1993, Kluck *et al.* 1993, Maeda *et al.* 1993, Maeda *et al.* 1995). It has further been suggested that oxidants may be responsible for part of the apoptotic process observed *in vitro* in several cell types, including thymocytes, chondrocytes, neurons, endothelial, mesengial and gonadal cells (Andersson *et al.* 1999, Dharmarajan *et al.* 1999, Gougeon 1996, Hiraishi *et al.* 1993, Hockenbery *et al.* 1993, Ishikawa and Kitamura 1999, Payne *et al.* 1995, Sugino *et al.* 2000, Tilly and Tilly 1995, Yoneda *et al.* 1995).

Also, the function of isolated ovarian cells *in vitro* is affected by plating density, for instance, FSH-induced steroidogenesis in rat and human granulosa cells. The production of estrogen and progesterone increases with increasing cell plating density to a certain level, after which it declines at yet higher cell plating densities (Bar-Ami and Gitay-Goren 1993, Davoren and Hsueh 1986). Furthermore, expression of insulin-like growth factor binding protein-5 (IGFBP-5) by ovine granulosa cells is regulated by cell culture density, with an increased expression of IGFBP-5 correlating with both high cell culture density and pronounced cell death (Monget *et al.* 1998).

## THE LH-SURGE

In preovulatory follicles, two principal physiological events occur as a consequence of the LH-surge; ovulation and luteinization. Luteinization is a process by which follicular granulosa and theca cells become nonmitotic and establish a specific, stable luteal cell phenotype. Biochemical changes in granulosa cells associated with luteinization include the marked and sustained induction of P450<sub>scc</sub>, the transient expression of progesterone receptors (PR) as well as dramatic and rapid decreases in mRNA for aromatase and 17- $\alpha$ -hydroxylase (converts pregnenolone and progesterone to different metabolites). These changes cause the conversion from estrogen production in the preovulatory follicle to progesterone synthesis in the corpus luteum (Clemens *et al.* 1998, Fitzpatrick *et al.* 1997, Richards 1994).

Events involved in ovulation include increased synthesis of prostaglandins as a consequence of induction by the LH-surge in preovulatory follicles of PGS-2 (prostaglandin endoperoxide synthase-2), the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins (Richards 1994). There is also an up-regulation of the expression of proteases thought to play critical roles in follicular rupture (Robker *et al.* 2000).

The finding that forskolin, a direct activator of adenylylcyclase, can mimic the effects of LH and FSH provides strong evidence for a primary role of cAMP and subsequent steps in the cAMP-dependent protein kinase (A-kinase) pathway in mediating theca and granulosa cell differentiation (Richards 1994, Clemens *et al.* 1998).

LH actions on ovarian target cells via cAMP-mediated pathways result in both acute and long-term increases in steroidogenesis. Acute actions include promotion of cholesterol transport into mitochondria, while long-term actions include stimulation of mRNA encoding components of the steroidogenic machinery (Chandrasekher *et al.* 1991).

## PROGESTERONE

Production of the steroid hormone progesterone within the ovary is critical for regulating specific ovarian functions, such as ovulation and luteinization (Brännström and Jansson 1989, Lydon *et al.* 1995, Mulac-Jericevic *et al.* 2000, Murphy 2000, Richards 1994, Rondell 1974, Rose *et al.* 1999, Rothchild 1981). The main source of progesterone in the non-pregnant female is the corpus luteum. During pregnancy, a high level of progesterone is produced by the placenta.

Physiologically, progesterone stimulates the proliferation of stromal cells of the uterus and mammary epithelium and regulates differentiation of the mammary gland and endometrium of the cycling uterus. Progesterone is also implicated in the pathological development and progression of breast and uterine malignancies (Gass *et al.* 1998).

Immunofluorescence studies have shown that granulosa cells with intense nuclear staining for PR after the LH-surge, exhibit intense staining of the mitochondrial P450<sub>scc</sub> protein before and after exposure to an ovulatory stimulus of LH (Natraj and Richards 1993). Thus, the ability of the granulosa cells to produce the ligand progesterone precedes the specific induction of PR (Chaffin *et al.* 1999).

Adrenal progesterone and corticosteroids have been shown to play an important role in modulating the preovulatory gonadotropin surge. Serum ACTH (adrenocorticotrophic hormone), corticosterone and progesterone are elevated on the day of proestrus in the rat prior to the onset of the LH-surge (Mahesh and Brann 1998). A rise in the levels of serum progesterone preceding an increase in serum LH has also been reported in women and monkeys. These data clearly indicate an important physiological role of progesterone, in addition to estradiol, in regulating the preovulatory surge of gonadotropins (Mahesh and Brann 1998). The stimulatory effect of progesterone on FSH secretion appears to have a direct effect on the FSH gene and can be blocked by the PR-antagonist RU 486 (O'Conner *et al.* 1997).

*In vivo* studies in humans indicate that progesterone stimulates its own production during the periovulatory and early to mid-luteal periods ("self-priming") (Natraj and Richards 1993).

Inhibitors of progesterone biosynthesis such as aminoglutethemide (Park and Mayo 1991) and epostane (Snyder 1984) as well as progesterone receptor antagonists can block LH induction of ovulation and luteinization *in vivo* and *in vitro* (Donath *et al.* 2000, Natraj and Richards 1993, Rose *et al.* 1999).

# THE PROGESTERONE RECEPTOR

## Regulation of expression

The progesterone receptor was initially characterized in the mammalian uterus and chick oviduct in the early 1970s. Since then, a variety of tissues have been shown to express PR or to be targets for progesterone action mediated via non-classical progesterone receptors, including the mammary gland, brain, sperm, blood vessel walls and the urinary tract (Brann *et al.* 1995, Graham and Clarke 1997, McDonnell 1995, Pinter *et al.* 1996, Shanker and Rao 1998). Identification of a PR protein in rat ovary was reported in 1979 (Schreiber and Erickson 1979, Schreiber and Hsueh 1979). Immunohistochemical localization of PR in the human ovary has revealed an expression of PR coinciding with the preovulatory LH-surge (Iwai *et al.* 1990). Expression of PR in granulosa cells is confined to the dominant follicle by the time of the LH-surge and the expression is localized exclusively to the nuclei (Iwai *et al.* 1990, Press and Greene 1988). After ovulation, the human PR persist in the luteinized granulosa cells and in the corpus luteum during early pregnancy. The theca interna and surrounding stromal cells express PR throughout the menstrual cycle. No expression of PR is found in non-dominant or atretic follicles (Iwai *et al.* 1990).

Primate studies have shown that an extended surge of LH, like that in the normal menstrual cycle (48-50 h), may be necessary for PR expression in, and luteinization of, granulosa cells in primate follicles (Chandrasekher *et al.* 1991).

In contrast to in human granulosa cells, the expression of PR mRNA and protein in rat granulosa cells is transient (Park and Mayo 1991, Natraj and Richards 1993). Little or no PR mRNA was found in ovaries isolated from rats prior to stimulation of LH receptors, either by endogenous LH or by exogenously administered hCG. However, using an immature rat model, it could be concluded that the PR mRNA was induced more than 20-fold in the immature ovary 5h after hCG administration and was down-regulated to near-basal levels by 12h after hCG administration. In adult, cycling rats, the expression of PR mRNA was transient and tightly coupled to the preovulatory LH-surge on the proestrous evening (Park and Mayo 1991). Theca cells or interstitial cells did not express detectable levels of PR mRNA. In cycling animals treated with pentobarbitone, to block the endogenous preovulatory surge of LH, no induction of PR mRNA on the proestrous evening was observed (Park and Mayo 1991). Basal concentrations of gonadotropins were not sufficient to induce rat PR, but elevated levels of gonadotropins were required. In contrast to humans, no PR expression was detected in corpus luteum, even in its functional stage during pregnancy, or in epithelial or stroma cells (Natraj and Richards 1993).

The appearance of PR protein, as determined by immunoblotting and indirect immunofluorescent staining, has been shown to follow a pattern similar to that of PR mRNA. PR increased by 3-5 h after *in vitro* administration of an ovulatory dose of LH, increased further by 7-9 h and was localized exclusively to nuclei (Natraj and Richards 1993).

Induction of PR mRNA and protein in rat granulosa cells depends on the stage of differentiation (Natraj and Richards 1993). The restricted induction of PR to a preovulatory granulosa cell phenotype suggests that previous exposure to estradiol is required for the differentiation of these cells, which then permits induction of PR in response to various agonists. Recent work by Clemens *et al.* has shown that, although 17- $\beta$ -estradiol can induce PR mRNA and activate PR promoter-reporter constructs in other cell types, the effects of 17- $\beta$ -estradiol in granulosa cells appear to be indirect. 17- $\beta$ -estradiol alone does not induce the expression of PR mRNA in preovulatory rat granulosa cells. Rather, induction of PR mRNA depends on the differentiation of granulosa cells in response to 17- $\beta$ -estradiol and

physiological amounts of FSH followed by exposure to LH that markedly increase cAMP (Clemens *et al.* 1998). Induction of PR mRNA by forskolin is blocked by an A-kinase inhibitor and cycloheximide but not by an antiestrogen (ICI 164,384). Thus, activation of the A-kinase pathway leads to the phosphorylation of some transcription factor(s) other than, or in addition to, estrogen receptors that is (are) critical for the transcription of the PR gene. This mechanism is selectively activated in differentiated granulosa cells possessing a preovulatory phenotype (Clemens *et al.* 1998).

The regulation of the intracellular amounts of PR takes place at the transcriptional and posttranscriptional levels. Both progestins and antiprogestins diminish the half-life of the receptor protein as well as the mRNA (Horwitz *et al.* 1995, Katzenellenbogen 1980, Read *et al.* 1988, Savouret *et al.* 1994). However, data have also been presented indicating a positive regulation of progesterone on its own receptor (Curry 1996; Ottander *et al.* 2000).

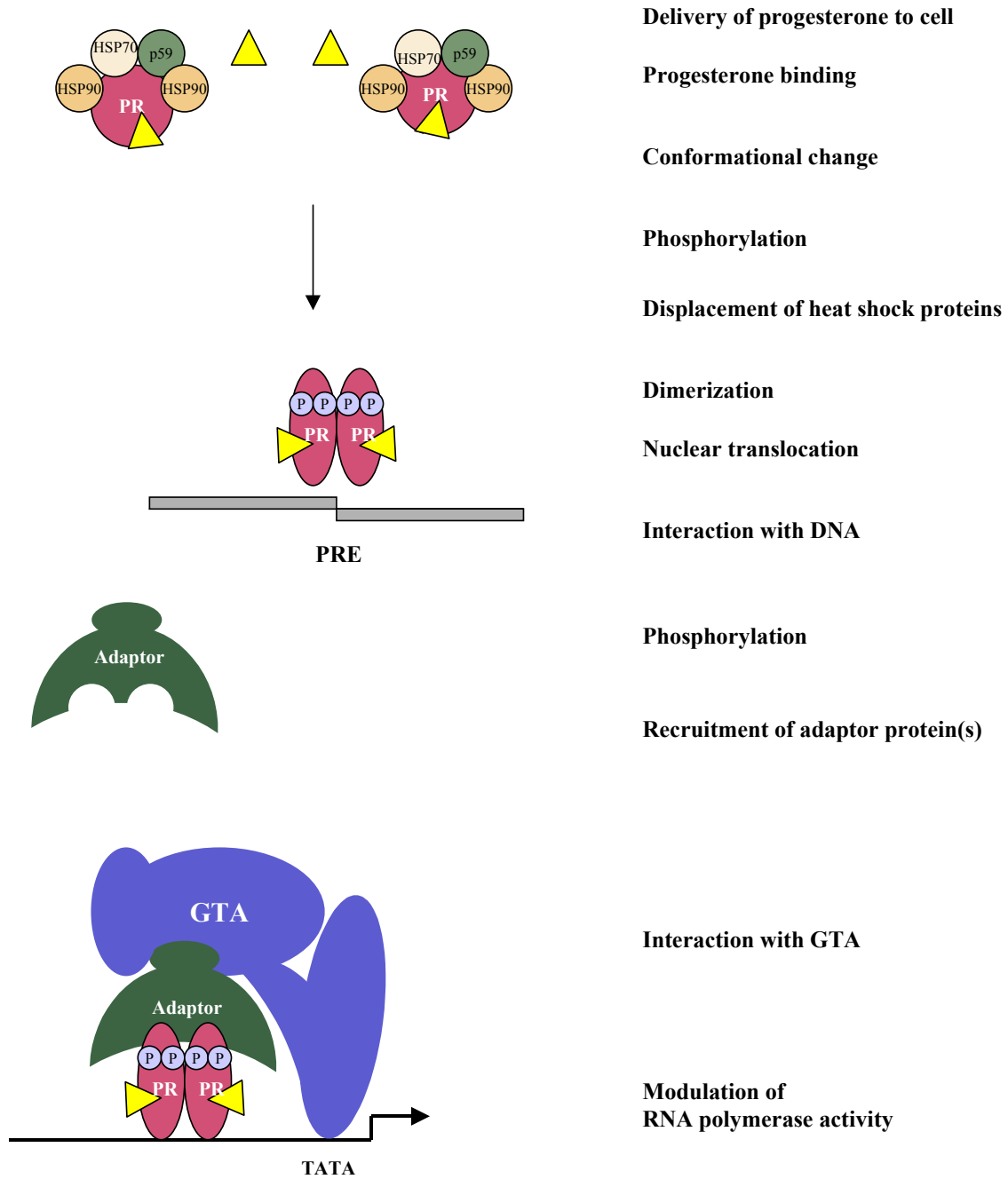
### **Structure and function**

The progesterone receptor is a ligand-activated transcription factor that belongs to the nuclear receptor superfamily of transcription factors (Evans 1988). Characteristic for transcription factors is that they mediate physiological effects by interacting with the host genome in such a way that specific gene expression is regulated (McDonnell 1995, Pinter *et al.* 1996).

In the absence of hormone, the transcriptionally inactive PR remains associated with a large complex of heat shock proteins in the nuclei of target cells (Smith *et al.* 1990). Upon hormone binding, the receptor dissociates from the heat shock protein complex, is phosphorylated, dimerizes and binds to progesterone responsive elements (PREs) within the regulatory regions of target genes (Figure 4). When bound to DNA, the PR dimer contacts components of the general transcription machinery, either directly or indirectly via cofactors such as coactivators and corepressors (Giangrande *et al.* 2000, Ing *et al.* 1992, McDonnell 1995).

In humans and rats, two isoforms of PR are described, hPR-A (94 kDa, 769 amino acids (aa), MW 95 000) and hPR-B (114 kDa, 933 aa, MW 120 000) (Ilenchuck *et al.* 1987, Lessey *et al.* 1983). In both species, PR-A is the dominant form expressed in the ovary (Misao *et al.* 1998, Ottander *et al.* 2000, Robker *et al.* 2000). cDNA encoding the rat PR in the ligand binding domain shows 80-85 % identity in the nucleotide sequence and 88-96 % identity in the aa sequence to human PR. The DNA binding domain is identical in the aa sequence to human PR cDNAs (Park and Mayo, 1991). Both forms of PR are derived from a single gene as a consequence of alternate initiation of transcription from distinct promoters (Kastner *et al.* 1990). The two isoforms have identical sequences in their carboxyl-terminal ligand binding domain (LBD) and in the centrally located DNA-binding domain (DBD). PR-A differs from PR-B only by lacking 164 amino acids of the extreme N-terminus (Kastner *et al.* 1990). In addition to these isoforms, a third isoform, hPR-C, even more truncated than hPR-A (60 kDa), has been identified in a human breast cancer cell line (T47D) (Wei *et al.* 1996). This isoform enhances progestin-induced transcription activity in the presence of PR-A and PR-B, but is inactive itself.

## THE MECHANISM OF ACTION OF PROGESTERONE



After D.P. McDonnell, *Trends Endocrinol Metab* 1995;6:133-138

**Figure 4.** The mechanism of action of progesterone.

While PR-A and PR-B have similar DNA- and ligand-binding affinities (Christensen *et al.* 1991), they have opposite transcriptional activities (Giangrande *et al.* 2000, Vegeto *et al.* 1993). In most contexts, hPR-B functions as an activator of progesterone-responsive genes, while hPR-A is transcriptionally inactive (Vegeto *et al.* 1993). In addition, hPR-A also functions as a strong transdominant repressor of hPR-B and human estrogen receptor- $\alpha$  (hER- $\alpha$ ) transcriptional activity in the presence of both PR agonists and antagonists (Giangrande *et al.* 1997, Leonhardt *et al.* 1998, McDonnell 1995). Finally, when bound to some progestin antagonists, PR-B, but not PR-A, can be converted to a strongly active transcription factor by modulating intracellular phosphorylation pathways (Mulac-Jericevic *et al.* 2000).

The precise mechanism underlying the differential activities of the two human PR isoforms is not yet fully understood. An inhibitory domain has been identified within the first 140 aa of hPR-A and shown to prevent hPR-A from functioning as a transcriptional activator (Giangrande *et al.* 1997). This aa sequence permits this receptor isoform to function as a transdominant repressor of heterologous steroid receptor transcriptional activity. Deletion of the N-terminal 140 aa (inhibitory domain) from hPR-A results in a receptor mutant that is functionally indistinguishable from hPR-B (Giangrande *et al.* 1997). Recent structure-function studies of the two receptor isoforms suggest that hPR-B contains three specific activation functions (AF-1, AF-2 and AF-3), whereas hPR-A contains only two (AF-1 and AF-2) (McDonnell 1995). AF-1, located in the amino terminus, and AF-2, located in the carboxyl terminus, are common to both hPR-A and hPR-B. The third putative activation function, AF-3, is located within the B upstream sequence, a region which is absent in hPR-A (Sartorius *et al.* 1994). In addition, sequences within hPR-A which contain an inhibitory domain inhibit both AF-1 and AF-2, but not AF-3 (Hovland *et al.* 1998).

In conclusion, these results support the hypothesis that hPR-A, like hPR-B, contains all of the sequences necessary for proper transcription activity. However, hPR-A is transcriptionally inactive, because in the absence of AF-3, the inhibitory domain prevents AF-1 and/or AF-2 from activating transcription. It thus seems that the role of AF-3 is to override the inhibitory function of the inhibitory domain, thereby allowing hPR-B to activate transcription (Giangrande *et al.* 1997, Giangrande *et al.* 2000, Hovland *et al.* 1998).

To be functionally active, the PR need to dimerize and for dimerization to occur both PR partners require ligand binding (Leonhardt *et al.* 1998). Three different isoforms are the result of PR dimerization; PR-A/PR-A, PR-B/PR-B and PR-A/PR-B.

## ANTIPROGESTINS

Antiprogestins (progesterone receptor antagonists) have been used experimentally for a long time to study the mechanisms of PR action (DiMattina *et al.* 1986, Horwitz 1995, Rose *et al.* 1999, Schoonen *et al.* 1998). Progestin agonists and antagonists appear to contact overlapping, but not identical, aminoacids in the ligand-binding domain (Cadepond *et al.* 1997, Gass *et al.* 1998, Leonhardt *et al.* 1998). A central property of PR antagonists is the induction of a conformational change in PR distinct from that induced by hormone agonists (Gass *et al.* 1998).

Leonhardt and colleagues have presented studies where the ligand binding domain of both hPR-A and hPR-B has been used to study transcription after homodimerization and mixed ligand heterodimerization of human progesterone receptors *in vivo* by a mammalian-two-hybrid assay. Without making the distinction between PR-A and PR-B-forms, the authors concluded that only agonist homodimers were able to initiate transcription, whereas antagonist homodimers were able to bind to DNA (PREs) but not to initiate transcription

since they induced an altered conformational change in the ligand binding domain that did not permit PR interaction with coactivators required to transcribe the receptor signal into a transcriptional response.

Interestingly, when cells are treated with both agonist and antagonists, heterodimers can be formed between PR bound to antagonist and PR bound to agonist. However, these heterodimers do not efficiently bind to PREs and thus appear to be inactive. This may be one reason for the very high potency of antiprogestins, difficult to explain by pure competition for the PR (Leonhardt *et al.* 1998).

### **RU 486 and Org 31710**

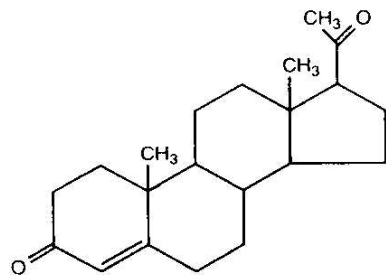
RU 486 (Roussel-Uclaf 38486; generic name, mifepristone) has received much clinical attention as a drug used for emergency postcoital contraception as well as for voluntary early pregnancy termination (Bygdeman *et al.* 1997, Cadepond *et al.* 1997, Croxatto *et al.* 1998, Task force on postovulatory methods of fertility regulation 1999). The main structural characteristic of RU 486 is the phenyl-aminodimethyl group perpendicularly grafted to the 11 $\beta$ -position of the steroidal skeleton (Cadepond *et al.* 1997). In addition to interaction with progesterone receptors, RU 486 is capable of interfering with glucocorticoid receptors (Cadepond *et al.* 1997, Kloosterboer *et al.* 1994), sometimes making it difficult to distinguish between antiglucocorticoid and antiprogestagen effects.

The effects of RU 486 on follicular development and ovulation could be either direct on the ovary or indirect via the hypothalamic pituitary axis, or both. According to Bygdeman *et al.*, the general consensus of a number of studies is that the preovulatory increase in progesterone of ovarian origin reinforces the positive feedback of oestrogen in triggering the midcycle LH-surge and that the inhibitory effect of antiprogestins on ovulation is mediated by a blocking effect of progesterone on the pituitary level (Bygdeman *et al.* 1997). *In vivo* studies in cynomolgus monkeys show that inhibition of ovulation by RU 486 occurs mainly at the level of the hypothalamus, with possible additional effects on the granulosa cell function and alterations of LH bioactivity (Heikinheimo *et al.* 1995).

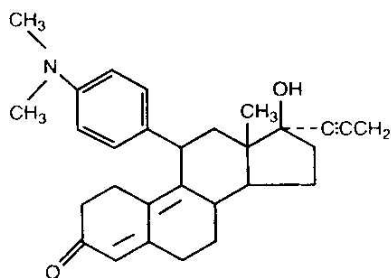
*In vitro* studies with human granulosa cells have demonstrated a dose-dependent decrease by RU 486 of the activity of 3 $\beta$ -HSD (converts pregnenolone to progesterone) and suppression of progesterone production (DiMattina *et al.* 1986). In addition, RU 486 may directly inhibit the activity of 17- $\alpha$ -hydroxylase, but does not directly inhibit aromatase activity or granulosa cell estradiol production *in vitro* (DiMattina *et al.* 1987).

Org 31710 (Organon 31710) is a highly selective antiprogesterin with no other known hormonal interactions except for weak androgenic and antiandrogenic activities (Kloosterboer *et al.* 1994, Hurd *et al.* 1997).

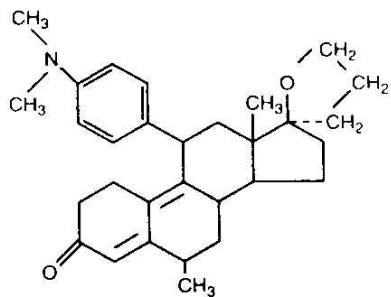
A relative decrease in antiglucocorticosteroid activity has been obtained with a tetrahydrofuran ring at the C17 $\alpha$ / $\beta$  position (Cadepond *et al.* 1997).



**Progesterone**



**RU486**



**Org31710**

**Figure 5.**  
*A schematic illustration showing the structures of progesterone and the two progesterone receptor antagonists RU 486 and Org 31710.*



## LEPTIN

More than 40 years ago, it was proposed that some unidentified signalling mechanism between the body's fat depots and the brain regulates food intake (Kennedy 1953). Parabiosis experiments in the late fifties and early seventies suggested a blood-borne factor to be the signaller (Coleman 1973, Hervey 1958). Lean rats were shown to starve to death when their circulatory systems were surgically joined to rats rendered obese by a lesion in the ventromedial hypothalamus. It was suggested that the lean animals starved due to exposure to a factor over-produced in the obese rats. This hypothesis was corroborated by Coleman and colleagues by using two different strains of genetically severely obese mice (*ob/ob* and *db/db*). The animals' circulatory systems were joined to those of wild-type animals or to each other. These studies showed that the *ob/ob* mice lacked a blood-borne anorexic factor, whereas the *db/db* mice were resistant to the effect of this factor, suggesting that the *db* gene encoded the receptor for the factor.

However, it was not until almost twenty years later, in 1994, that the gene encoding the anorexic factor was cloned (Zhang *et al.* 1994). The polypeptide was given the name leptin from the greek word léptos, meaning "thin". Since then, evidence has been presented by many investigators that leptin acts as an afferent signal in a feedback loop from the adipose tissue to the hypothalamus in the regulation of energy balance (Campfield *et al.* 1995, Halaas *et al.* 1995, Pelleymounter *et al.* 1995).

### Leptin structure

Leptin is a 16 kDa protein (167 aa), mainly produced by adipocytes (Zhang *et al.* 1994) but also found in the placenta during pregnancy (Hoggard *et al.* 1997) as well as in the gastric epithelium and heart (Bado *et al.* 1998, Green *et al.* 1995). Serum leptin levels in humans as well as in rodents correlate with the amount of body fat, high levels of leptin being associated with high amounts of body fat. However, at the same body mass index (BMI), there are great interindividual variations in circulating leptin levels (Considine *et al.* 1996a, Maffei *et al.* 1995).

Examples of factors involved in the regulation of leptin, besides body fat mass, include sex, hormonal stimulation and nutritional status (Dallongeville *et al.* 1998). The higher serum leptin levels in women relative to men are partially the result of the increased total body fat content in women and partially the result of the differences in body fat distribution, with relatively more subcutaneous versus omental adipose tissue in women. In addition, sex hormones contribute to these sex differences in leptin levels. Androgens have a potent suppressive effect on leptin expression in adipocytes and are negatively correlated to plasma leptin levels (Dallongeville *et al.* 1998).

Leptin levels are increased by hormones such as insulin (Saladin *et al.* 1995), glucocorticoids (Mac Dougald *et al.* 1995), estrogens (Mannucci *et al.* 1998, Shimizu *et al.* 1997), tumour necrosis factor alpha (Mantzoros *et al.* 1997a) and interleukin-1 (Janik *et al.* 1997). A leptin-regulated inhibitor of proximal leptin signalling has been suggested in SOCS-3 (Bjorbaek *et al.* 1999).

Leptin circulates in the blood in both a free form and bound to other proteins. The plasma binding proteins have not yet been completely characterized but include a secreted form of the leptin receptor (Houseknecht *et al.* 1996, Patel *et al.* 1999, Schubring *et al.* 2000, Sinha *et al.* 1996).

## Leptin receptors

The leptin receptor belongs to the class I cytokine receptor family (Tartaglia *et al.* 1995). Multiple splice variants of the leptin receptor are produced from the same gene and differ in the length of the intracellular domain (Cioffi *et al.* 1996, Lee *et al.* 1996, Tartaglia *et al.* 1995). The long leptin receptor is predominantly expressed in the hypothalamus and signals via the JAK/STAT and the MAPK pathways (Houseknecht and Portocarrero 1998). This isoform is considered to be the principal signalling isoform, in line with studies on other members of the cytokine receptor superfamily that have shown that both box 1 and box 2 are required for efficient signal transduction (Taniguchi 1995).

The short isoforms of the leptin receptor are widely expressed and are, although substantially weaker than the long isoform, still able to transmit some signalling (Bjorbaek *et al.* 1997). The significance of truncated leptin receptor signalling for leptin biology *in vivo* is unknown (Bjorbaek *et al.* 1997, Cohen *et al.* 1996, Ghilardi *et al.* 1996, Murakami *et al.* 1997). It has been proposed that short isoforms may act as dominant negative receptors (Vaisse *et al.* 1996). However, transfection experiments have shown that the long isoform of the leptin receptor is relatively resistant to dominant negative repression by truncated receptor isoforms (White *et al.* 1997). One isoform lacks the intracellular and transmembrane domains and is thought to act as a soluble receptor binding leptin in circulation (Lee *et al.* 1996).

## Central effects of leptin

To reach its central site of action, leptin crosses the blood-brain barrier via a saturable transport system, which has been suggested to involve the short form of the leptin receptor, which is highly expressed in the choroid plexus (Friedman and Halaas 1998) and on capillary endothelia throughout the brain (Bjorbaek *et al.* 1998, Golden *et al.* 1997).

Leptin acts both by suppressing food intake and by stimulating energy expenditure, including thermogenesis (Campfield *et al.* 1995, Halaas *et al.* 1995). The suppression of food intake is supposed to involve suppression of neuro-peptide Y (NPY) expression and secretion in the arcuate nucleus, since NPY is a strong stimulator of food intake and is involved in the regulation of various pituitary hormones (Stephens *et al.* 1995). Interestingly, NPY plays an inhibitory role in the GnRH-LH/FSH axis. Thus, leptin may counteract this inhibitory activity, favouring sex-steroid synthesis and secretion (Schubring *et al.* 2000).

In addition to modulation of NPY activity, a variety of neuroendocrine signals are suggested to be regulated by leptin (Håkansson *et al.* 1998, Kristensen *et al.* 1998), indicating a multiplicity of neuroendocrine targets for leptin interaction.

The role of leptin in human obesity is not yet fully understood, as mutations in the human leptin gene (Carlsson *et al.* 1997, Considine *et al.* 1995, Maffei *et al.* 1996, Montague *et al.* 1997) as well as in the human leptin receptor (Clément *et al.* 1998, Considine *et al.* 1996b, Gotoda *et al.* 1997, Matsuoka *et al.* 1997) are rare. Leptin concentrations in the cerebrospinal fluid (CSF) are strongly correlated in a nonlinear manner to plasma levels and BMI. However, in obesity there is a less important increase in the CSF leptin levels relative to the increase in serum leptin. A higher CSF/serum leptin ratio is found in lean compared to obese subjects, suggesting a reduced efficiency of brain leptin transport and a relative leptin deficiency in the central nervous system in obese individuals (Caro *et al.* 1996, Schwartz *et al.* 1996).

## Leptin and reproduction

Body weight has long been known to correlate to fertility (Frisch 1978, Rich-Edwards *et al.* 1994, Zaadstra *et al.* 1993). Too low as well as too high percentages of body fat are known causes of female infertility (Reid and Van Vugt 1987) and the amount of body fat is also a determinant of menarche (Frisch and McArthur 1974). Weight loss in anovulatory obese women results in significantly increased pregnancy and ovulation rates (Clark *et al.* 1995).

Leptin has been suggested as a signal link between fat stores and the central reproductive system (Ahima *et al.* 1997, Barash *et al.* 1996, Bray 1996, Chehab *et al.* 1996, Chehab *et al.* 1997, Cheung *et al.* 1997). Genetically obese mice that lack leptin are infertile (Ingalls *et al.* 1950). Diet restriction of the *ob/ob* female mice causes weight reduction, but fails to correct sterility. However, administration of leptin to these animals restores their fertility (Chehab *et al.* 1996).

In rodents, there are indications that leptin modulates the reproductive endocrine system at the hypothalamus-pituitary level as well as at the ovarian level (Ahima *et al.* 1996, Erickson *et al.* 1996, Zachow and Magoffin 1997). In humans, the importance of leptin for reproduction is indicated by the absence of pubertal development in women with a mutated leptin receptor (Clement *et al.* 1998). Correlations between leptin and estrogen during the menstrual cycle have been reported and found to be consistent with reported perimenstrual variations in food craving and consumption (Mannucci *et al.* 1998). During pregnancy, maternal leptin serum levels continuously increase from 6-8 weeks up to 38-40 weeks of pregnancy and decrease dramatically after birth (Butte *et al.* 1997).

## AIMS OF THE THESIS

The susceptibility of the preovulatory follicle, exposed to the LH-surge, to undergo apoptosis seems to be dramatically reduced compared to the susceptibility of follicles of previous stages of differentiation.

The general aim of this thesis was to investigate cellular mechanisms involved in regulation of luteinizing granulosa cell susceptibility to apoptosis.

The specific aims of the studies were

- I To characterize the in vitro conditions under which the experiments would be performed, with special focus on cell concentration.
- II To elucidate whether LH receptor-mediated differentiation of the ovarian follicle makes the granulosa cells of the follicle less susceptible to apoptosis.
- III To investigate whether progesterone receptor signalling is involved in regulating the susceptibility to apoptosis in LH receptor-stimulated preovulatory granulosa cells.
- IV To investigate if leptin, apart from its central regulation of gonadotropin release, is also capable of eliciting biological responses at the ovarian level affecting granulosa cell survival, which might shed light on the cause of obesity-related infertility.

# GENERAL METHODOLOGICAL CONSIDERATIONS

The methods used in this thesis are described in detail in the Materials and Methods sections of the individual papers. A more general discussion of some of the methods is presented in the following section.

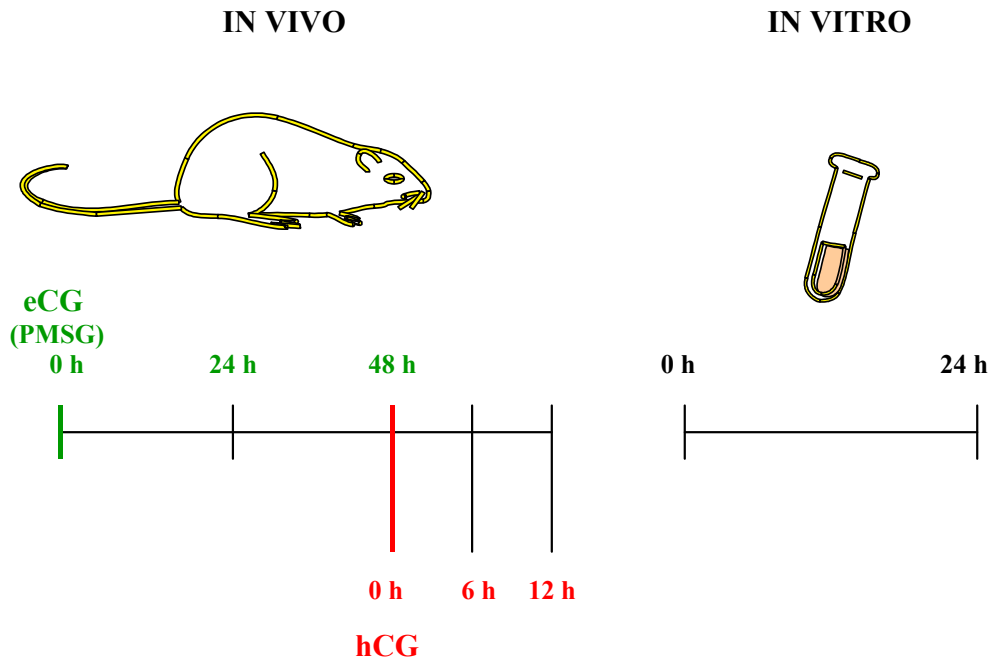
## EXPERIMENTAL DESIGN

Some of the experiments in this thesis were performed using granulosa cells directly isolated from ovaries with no subsequent incubation before analysis. In most experiments, however, the granulosa cells were subjected to an *in vitro* situation during culture in serum-free medium for various periods of time. The reason for using *in vitro* studies was to be able to study direct effects of different manipulations on granulosa cells.

Granulosa cells obtained from rats as well as from women have been used. Human granulosa cells were collected from women under hormonal treatment prior to IVF/ET (In Vitro Fertilization/Embryo Transfer). As these cells have been exposed to FSH receptor stimulation as well as LH receptor stimulation *in vivo*, it is more correct to refer to them as luteinizing granulosa cells. In Paper II, where PR-mediated effects in rat granulosa cells were studied, animals were treated *in vivo* with eCG/PMSG (equine Chorionic Gonadotropin/Pregnant Mare's Serum Gonadotropins), stimulating the FSH receptor, and with hCG (human Chorionic Gonadotropin) to mimic the endogenous LH-surge with concomitant stimulation of the LH receptor. Thus, the human and the rat model are very much alike concerning experimental design.

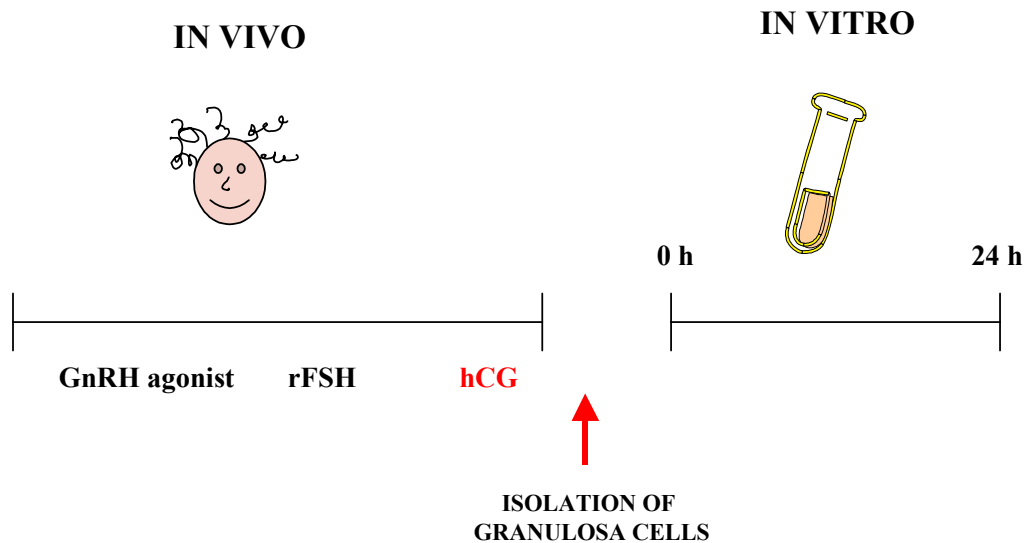
It would obviously be of greatest interest to conduct studies on human granulosa cells in varying stages of differentiation, isolated from women with spontaneous menstrual cycles. Unfortunately, these cells are very hard to collect in sufficient amounts to perform the kind of *in vitro* experiments done in this thesis. However, in Paper IV, follicular cells from a few women with regular menstrual cycles were collected for subsequent mRNA analyses.

## RAT MODEL



**Figure 6.** Immature rats were treated with eCG/PMSG for various periods of time to induce follicular development. In addition, some animals received hCG-treatment to mimic the endogenous LH-surge. In some experiments cell analysis was performed directly after isolation of the cells. In other experiments the cells were subjected to incubation for 24 h in serum-free medium.

## HUMAN MODEL



**Figure 7.** Human luteinizing granulosa cells were collected from women undergoing hormonal treatment prior to IVF. Before follicular puncture, the women had been treated with a GnRH (Gonadotropin Releasing Hormone)-agonist for three weeks to down-regulate the endogenous gonadotropin stimulation of follicular development. Then recombinant FSH was administered to induce follicular maturation of several follicles. When the follicles had reached a satisfying size (as determined by ultrasonography), an ovulation-inducing dose of hCG was given. Follicle puncture was performed approximately 36 h later.

## ISOLATION OF GRANULOSA CELLS

Human granulosa cells were obtained in connection with oocyte retrieval, using vaginal ultrasound-guided puncture of preovulatory follicles. For some follicles, where an egg was not found after the puncture of the follicle, the follicle was rinsed with medium until the egg was obtained. From these follicles, a large amount of granulosa cells was often obtained. After the egg from each follicle was secured, the remaining granulosa cells in the medium were collected. Granulosa cells from several patients were pooled in each experiment.

To get rid of erythrocytes, cell suspensions were rinsed in culture medium and subjected to isotonic Percoll<sup>®</sup>-centrifugation, with subsequent washing of the granulosa cells in culture medium again.

Rat granulosa cells were isolated by puncture of preovulatory follicles in isolated ovaries.

## INTERCELLULAR COMMUNICATION BETWEEN FOLLICULAR CELLS

The ovarian follicle is composed of three different cell types; the oocyte, which is enclosed by the granulosa cells, and outermost, separated from the granulosa cells by a basal lamina, the theca cells (Motta *et al.* 1997). Granulosa cells interact with theca cells and the oocyte in an intricate manner, not yet fully elucidated (Eppig *et al.* 2000, Vanderhyden *et al.* 1992). Thus, when isolating granulosa cells for *in vitro* use, these cells are pulled out of their physiological context.

Treatments with FSH, LH/hCG or insulin-like growth factor-I (IGF-I) are ineffective in preventing spontaneous apoptosis in granulosa cells, despite their apoptosis-suppressing action in cultured ovarian follicles (Billig *et al.* 1996). Also, estrogen production in the granulosa cells requires the presence of theca cells as providers of the aromatase substrate (Hsueh *et al.* 1984). In spite of these limitations, the model has generated a considerable amount of knowledge, also valid *in vivo*, as shown by several investigators (Hsueh *et al.* 1984, Hsueh *et al.* 1994, Richards 1994).

## THE CULTURE MEDIUM

In Papers I-III, bovine serum albumin (BSA) was added to the culture medium. The reasons for this were to mimic the extracellular milieu and to prevent hormones added to the culture medium from adhering to the incubation vials.

However, one has to bear in mind that albumin itself may, to some extent, bind hormones added to the culture medium, thereby interfering with the amount of ligand available. In Papers II and III we raised the question whether progesterone added to the culture medium could be bound to the BSA in the medium, thereby preventing the access of progesterone into the cells. To address this question, experiments were done where progesterone was added to culture medium with or without supplementation with BSA. The degree of DNA fragmentation in these cultures was similar, demonstrating that, in this context, BSA did not affect the endpoints used in Papers I-III.

## METHODS FOR APOPTOSIS DETECTION

### DNA fragmentation

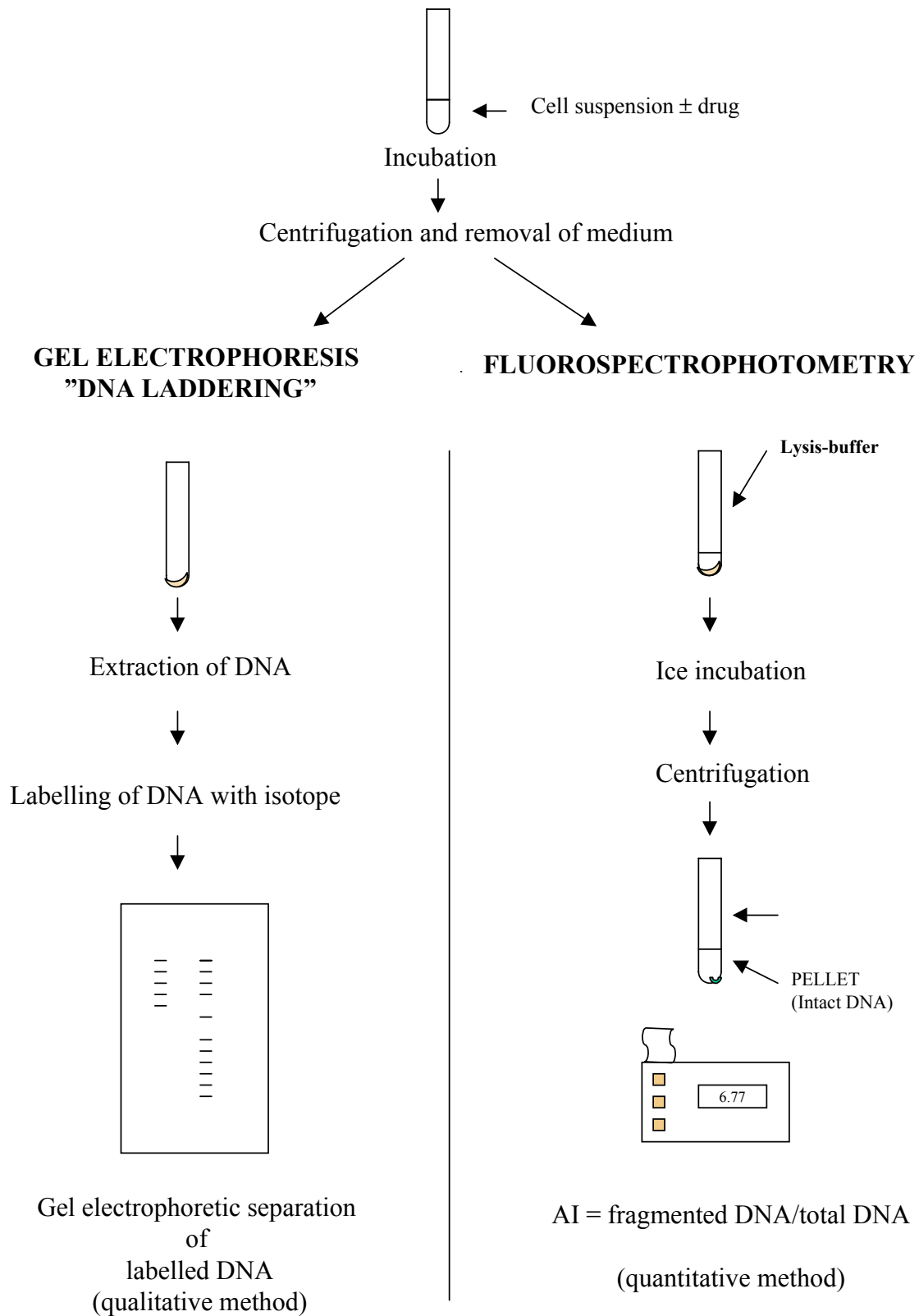
Internucleosomal DNA fragmentation has been shown by a multitude of investigators to be a typical sign of apoptotic cell death (Billig *et al.* 1993, Tilly and Hsueh 1993, Wyllie 1980). In this thesis, two different methods were used to study DNA fragmentation. Using a fluorospectrophotometric method, DNA was isolated from granulosa cells and then subjected to centrifugation. This provides a supernatant fraction with low molecular weight DNA (fragmented DNA), and a pellet comprising high molecular weight DNA. DNA content in the two fractions was measured using fluorospectrophotometry after labelling the DNA with Hoechst's dye. This dye binds selectively to DNA (Labarca and Paigen 1980). The fraction of low molecular weight DNA of total DNA (supernatant +pellet) was calculated and presented as the Apoptotic Index (AI).

A limitation of the fluorospectrophotometric method is that the nature of the fragmented DNA cannot be established. Thus, the fragmented DNA can be of necrotic as well as of apoptotic origin. This emphasizes the need to combine this quantitative method with a qualitative method, visualizing the actual appearance of the fragmented DNA. For this purpose, gel electrophoretic separation of isotope-labelled DNA was used. In case of internucleosomal DNA fragmentation, a typical "ladder" was seen on the gel, representing DNA fragments of 180-200 basepairs and multiples thereof.

### Caspase-3 activity

In addition to internucleosomal DNA fragmentation, other characteristic events typical of apoptosis can be used as endpoints. We chose to study caspase-3 activity, since this is a well-known apoptosis-regulated event, shown to be involved in human as well as in rat granulosa cell apoptosis (Boone and Tsang 1998, Izawa *et al.* 1998). All known caspases possess an active-site cysteine and cleave a specific aa sequence after aspartic acid residues (D). The proteolytic assay used in this thesis is specific for cleavage of the DEVD-sequence. A caspase's distinct substrate specificity is determined by the four residues amino-terminal to the cleavage site (Hengartner 2000). The synthetic substrate used in this assay was acetyl-Asp-Glu-Val-Asp-7-amido-4-methyl-coumarin (Ac-DEVD-AMC). AMC is a fluorescent compound, but fluorescence is blocked until the compound is cleaved off from the rest of the substrate. Cleavage of this substrate may also, to some extent, be mediated by caspase-7. However, caspase-3 is considered as the main effector caspase in most cells, both with regard to abundance and its crucial role in degrading important "death substrates" (Hengartner 2000). Even though a minor portion of the DEVD-cleavage measured in the study may be due to other caspases than caspase-3, the increased proteolytic activity is still related to the apoptotic process.





*Figure 8. Methods used to detect DNA fragmentation.*

## **INDIRECT MEASUREMENTS OF REACTIVE OXYGEN SPECIES**

The short half-life of most reactive oxygen species in biological systems does not permit their detection and quantification. Therefore, detection of reactive oxygen species relies on indirect measurement of modified targets (Ischiropoulos 1998). Biological targets that have been utilized for detection of oxidative modification include lipids, proteins, thiols and DNA. However, reactive oxygen species react with more than one biological target and, since the concentration of biological targets varies among cells, it is difficult to predict which target will be preferentially modified. Another method of detecting reactive oxygen species is the use of “reporter” compounds that will be oxidized by reactive species to either chromogenic, fluorescent or luminiscent products.

In this thesis, we have used three different methods for indirect measurement of free radicals, which will be briefly discussed.

### **Nitrite assay**

In this assay, the concentration of nitrite ( $\text{NO}_2^-$ ) in the culture medium after 24 h of incubation was used as an index for cellular production of nitric oxide. At the end of the incubation, the culture medium was mixed with Griess reagent (forms a purple azo derivative in the presence of nitrite), after which the mixtures were measured spectrophotometrically at 543 nm. Prior to the addition of Griess reagent, the culture medium was decolourised by the addition of 10% ZnCl to avoid interference with the absorbance measurements.

### **Superoxide dismutase (SOD)-inhibitable reduction of cytochrome c**

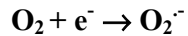
In this method, cytochrome c was added to the culture medium at the start of incubation. To some samples, SOD was added as well. Reduction of the ferrous atom of cytochrome c will cause a change in the colour of the medium. If this colour-shift is not present in the tubes where SOD was included, this indicates that superoxide ions have been present in the medium during incubation. The absorbance of the medium was measured spectrophotometrically at 550 nm. Culture medium without phenol red was used to avoid interference with the absorbance measurements.

### **Hydroxylation of deoxyguanosine to 8-hydroxy-deoxyguanosine**

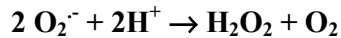
Formation of hydrogen peroxide or extracellular formation of hydroxyl radicals was determined by measurement of hydroxylation of deoxyguanosine to 8-hydroxydeoxyguanosine. High performance liquid chromatography with electrochemical detection was used for detection of 8-hydroxydeoxyguanosine.

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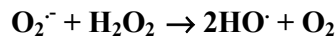
Superoxide ions are formed by one electron reduction of molecular oxygen:



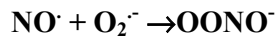
Superoxide ions may undergo several reactions, including dismutation to hydrogen peroxidase. This reaction is spontaneous, but is also catalyzed by superoxide dismutase (SOD):



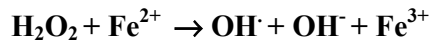
Superoxide can also react with hydrogen peroxide, forming the highly reactive hydroxyl radical



In the presence of nitric oxide, peroxynitrite may be formed



H<sub>2</sub>O<sub>2</sub> at low μM levels does not react with many biological targets at an appreciable rate. However, the reaction of H<sub>2</sub>O<sub>2</sub> with reduced divalent redox active metals, such as iron, can lead to the formation of strong oxidants via the Fenton reaction



*Figure 9. A schematic presentation of the generation of some reactive oxygen species.*

## DETECTION OF mRNA

### Ribonuclease protection assay (RPA)

Expression of the long and short leptin receptor isoforms in human ovarian cells was analysed by an RPA developed to differentiate between the isoforms. In the first step, RNA isolated from the cells was hybridized over night with a <sup>33</sup>P-labelled human leptin receptor cRNA probe. Subsequently, the samples were treated with RNase A and RNase T1, to digest unprotected fragments. Protected fragments were precipitated and separated on a denaturing polyacrylamide gel, which was then exposed on a PhosphorImager screen.

Using this method, only the short isoform of the leptin receptor was detected. Since this could be due to the sensitivity limit of the method, another set of experiments using RT-PCR was performed.

## **Reverse transcriptase polymerase chain reaction (RT-PCR)**

This method was used to analyse the expression of rat progesterone receptor (Paper II) and of human leptin and leptin receptors (Paper IV). PCR is a very sensitive method, allowing analysis of gene expression of rare messages from a small number of cells. Total RNA content in a tissue or cell suspension was extracted according to the guanidinium isothiocyanate procedure (Chomczynski and Sacchi 1987). The total RNA was then converted to cDNA by the action of reverse-transcriptase. The specific mRNA of interest was amplified by the use of primers binding only to the specific mRNA. By allowing this procedure to take place for several cycles, in which the specific product of interest is amplified, one can later easily detect the product. In Paper II, the PCR amplification was carried out in the presence of  $\alpha$ -<sup>32</sup>P-dCTP, thereby allowing autoradiography and later analysis by a PhosphorImager screen. In Paper IV, the PCR products were separated on agarose gels containing ethidium bromide, allowing visualization by UV-light.

## **CULTURE OF HUMAN LUTEINIZING GRANULOSA CELLS**

Granulosa cells obtained from women undergoing IVF-treatment have been exposed to high concentrations of gonadotropins *in vivo* and are more correctly referred to as luteinizing granulosa cells. These cells are initially less responsive to further treatment with gonadotropins *in vitro*, an effect likely to be due to partial desensitization at the receptor level (Hillensjö *et al.* 1985). This was the reason for pre-culturing the cells before addition of hormones in Paper IV. Testosterone was added to the culture medium as an aromatase substrate for estradiol production.

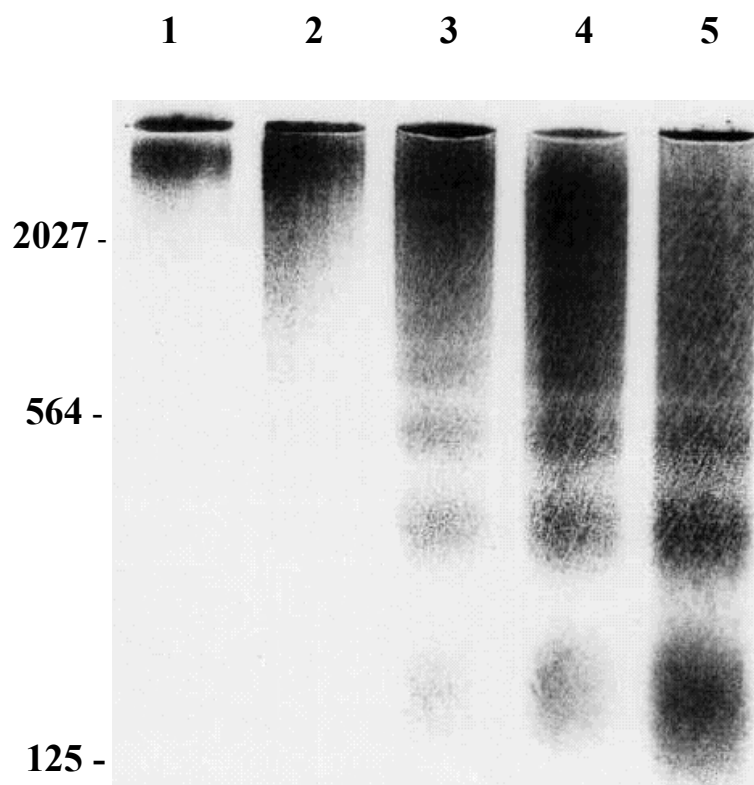
## RESULTS AND COMMENTS

In this section, the results of Papers I-IV are summarized and discussed. In addition, some unpublished data of relevance will be presented.

### Cell-concentration-dependent apoptosis

#### (Paper I)

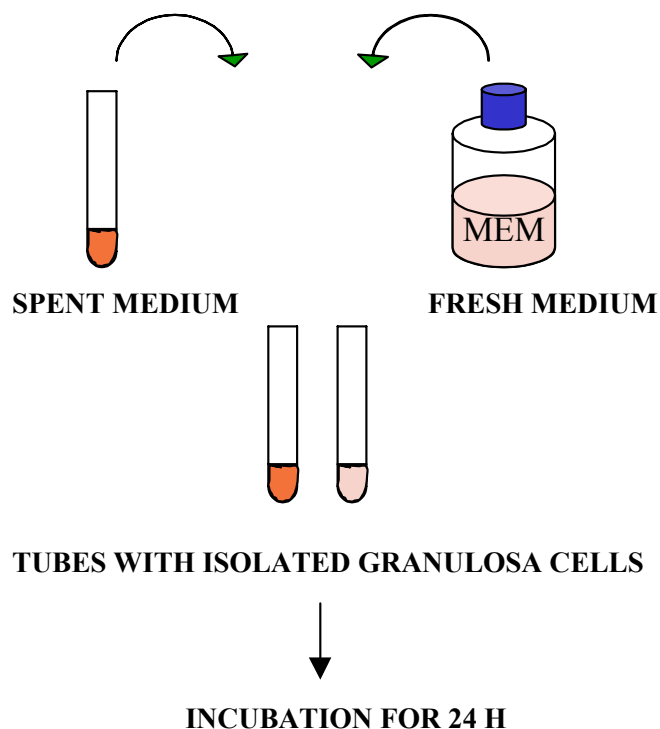
To be able to interpret *in vitro* data, it is important to characterize the *in vitro* conditions used. Cell concentration has been reported to affect the degree of apoptosis in various cell types (Bruckner *et al.* 1989, Ishizaki *et al.* 1993, Kluck *et al.* 1993, Maeda *et al.* 1993, Maeda *et al.* 1995). To evaluate the influence of this parameter in our culture system, we incubated rat granulosa cells with increasing cell concentrations (either as a varying number of cells in the same volume of medium or as a constant number of cells in varying volumes of medium). A cell-concentration-dependent apoptosis was obvious, with high cell concentrations corresponding to high degrees of internucleosomal DNA fragmentation.



**Figure 10.**

*Cell-concentration-dependent internucleosomal DNA fragmentation in rat granulosa cells, visualized by gel electrophoresis. Granulosa cells were collected from immature rats treated with PMSG for 48 h and incubated in serum-free medium in incubation tubes at different concentrations (million cells/0.5 ml): (1): 0.125 (2): 0.250 (3): 0.500 (4): 1.00 (5): 2.00. The left-hand margin indicates sizes of DNA fragments (basepairs), estimated by comparison of migration distances to a DNA ladder (Lambda DNA/Hind III). The same amount of DNA is loaded into each lane.*

Incubation for 24 h of freshly isolated granulosa cells (low cell concentration) with spent medium from 24 h cultures with high concentrations of granulosa cells, did not increase the degree of DNA fragmentation in the cells compared to in granulosa cells incubated in fresh medium. This finding argues against secretion of apoptosis-inducing factors from granulosa cells to the medium and/or change in pH and/or decrease in nutrients as causes of the cell-concentration-dependent apoptosis.

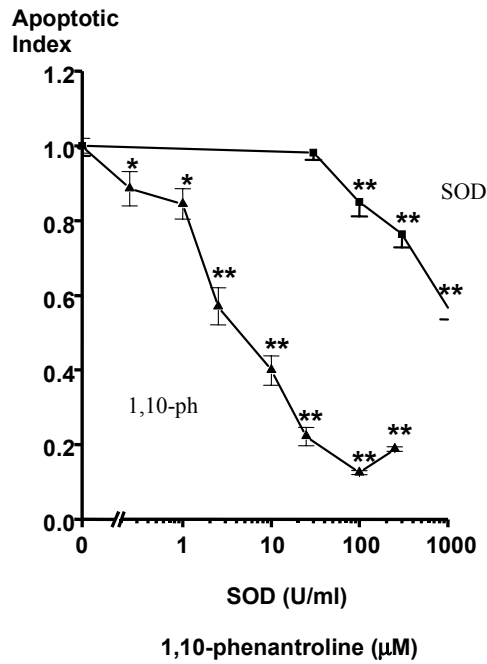


**Figure 11.**

*Culture of granulosa cells (low cell concentration) in either spent medium from high cell concentration cultures or in fresh medium. The degree of DNA fragmentation was similar in the two groups after 24 h incubation.*

Oxidants / free radicals are highly reactive compounds with only a transient existence, eliciting different detrimental effects in cells. To investigate if oxidants could be mediators of the cell-concentration-dependent apoptosis in our cultures, three different indirect measurements of oxidants were used. However, using these methods, we were not able to detect such agents in our cultures. Another way of indirectly detecting the existence of oxidants is to interfere with the production of oxidants. Indeed, addition to the culture medium of three different scavengers of oxidants (1,10-phenanthroline, superoxide dismutase (SOD), heparin) decreased the cell-concentration-dependent DNA fragmentation observed in the cultured granulosa cells. This suggests the involvement of oxidants in cell-concentration-dependent apoptosis.

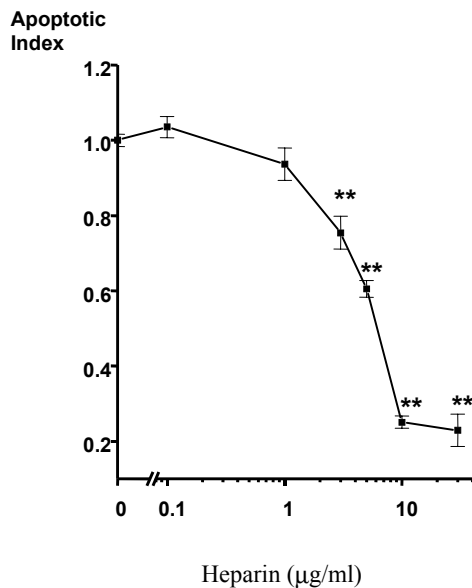
In conclusion, the data presented in this paper emphasize the importance of taking *in vitro* conditions into consideration before interpreting *in vitro* data. As a consequence of the findings, the same cell concentration was used in all subsequent experiments (500 000 cells/0.5 ml for experiments with rat granulosa cells and 100 000 cells/0.5 ml for experiments with human granulosa cells).



**Figure 12.**

*Inhibition of apoptosis in rat granulosa cells by 1,10-phenantroline or SOD.*

Granulosa cells were incubated ( $0.5 \times 10^6$  cells/tube, 24 h) in the absence or presence of 1,10-phenantroline (-▲-; 0.25 –250  $\mu\text{M}$ ) or superoxide dismutase (SOD) (-■-; 30-1000 U/ml). 1,10-phenantroline dose-dependently decreased DNA fragmentation with a maximal effect at 100  $\mu\text{M}$ , the inhibition at 0.25  $\mu\text{M}$  being statistically significant.  $n=6-12$  except for 1, 100 and 250  $\mu\text{M}$ , where  $n=3$ . SOD significantly inhibited DNA fragmentation at 100 U/ml compared to control.  $n=9-12$ , except for 30 U/ml, where  $n=6$ . DNA fragmentation was assayed fluorospectrophotometrically. (\* :  $P<0.05$  and \*\* :  $P<0.01$  compared to control).



**Figure 13.**

*Inhibition of apoptosis in rat granulosa cells by heparin.* Granulosa cells were isolated and incubated ( $0.5 \times 10^6$  cells/tube, 24 h), with or without addition of heparin (0.1  $\mu\text{g/ml}$  – 30  $\mu\text{g/ml}$ ) to the culture medium. Heparin dose-dependently decreased the apoptotic DNA fragmentation, a significant effect being achieved at 3  $\mu\text{g/ml}$  and maximal effect at 30  $\mu\text{g/ml}$ . DNA fragmentation was assayed fluorospectrophotometrically. (\*\* :  $P<0.01$  compared to control).  $n=6-15$ .

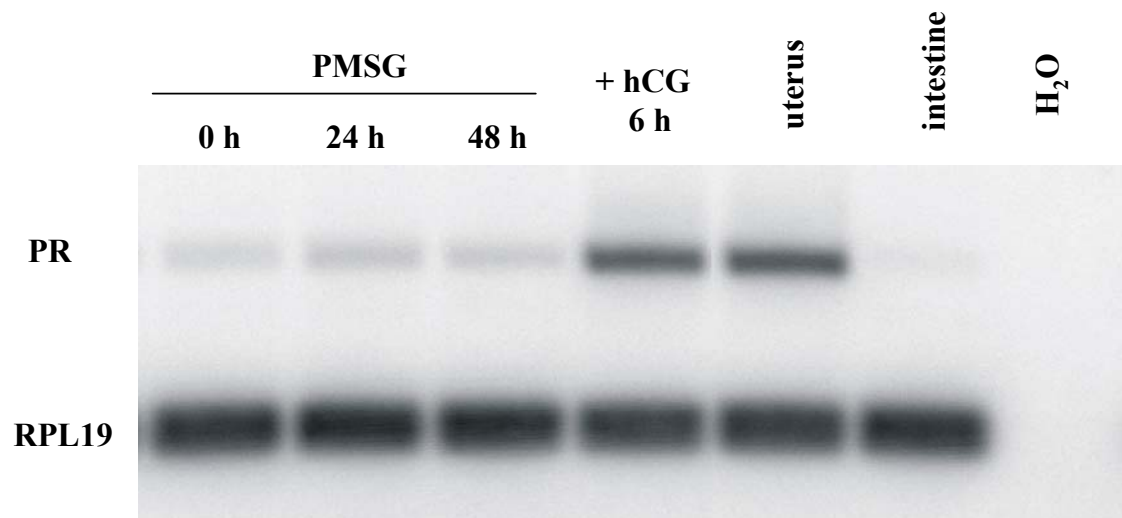
# Progesterone receptor-mediated inhibition of apoptosis in rat luteinizing preovulatory granulosa cells

## (Paper II)

Preovulatory follicles responding to the LH-surge seem to be rescued from the apoptotic programme operating at a high rate at all other preceding stages of ovarian follicular development. This made us pose the question whether stimulation of the LH receptor *in vivo* confers a decreased susceptibility to apoptosis in granulosa cells. To address this question, experiments were performed, where immature rats were treated with eCG to induce follicular maturation with or without subsequent treatment with hCG to mimic the endogenous LH-surge, causing stimulation of LH receptors.

The degree of internucleosomal DNA fragmentation was studied in granulosa cells isolated from rats subjected *in vivo* to different hormonal treatments. It was shown that treatment with eCG *in vivo* confers a decreased degree of apoptosis in granulosa cells, which was even more pronounced after treatment with hCG *in vivo*.

Characteristic of rat granulosa cells exposed *in vivo* to the LH-surge is a high production of progesterone coinciding with a transient expression of PR in these cells. RT-PCR verified that the expression of PR mRNA increased dramatically after hCG-treatment. Also, the progesterone production increased dramatically after stimulation with hCG *in vivo*, measured as the accumulated amount of progesterone in culture medium after 24 h of incubation.



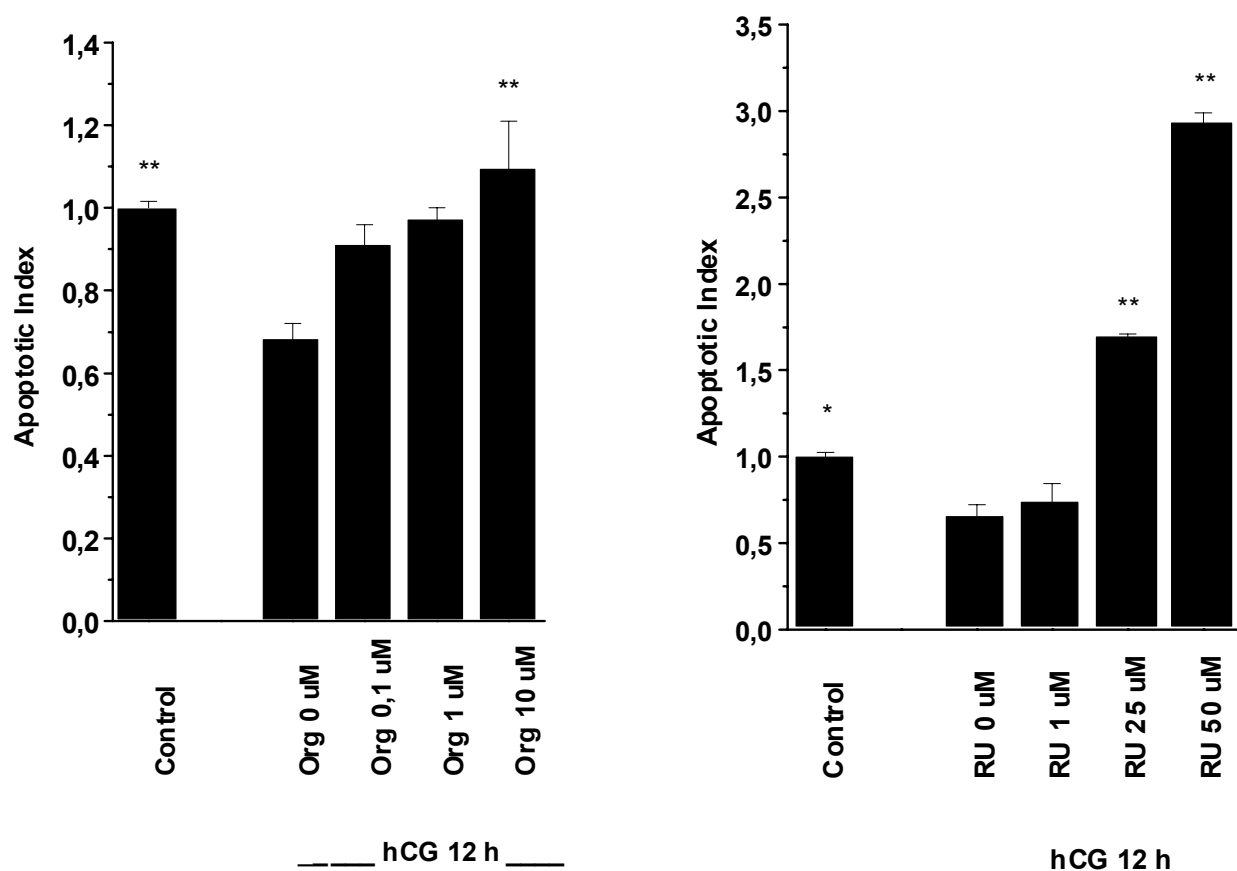
**Figure 14.**

*Effect of in vivo differentiation on progesterone receptor mRNA expression in rat granulosa cells. Representative figure showing RT-PCR detection of progesterone receptor mRNA, visualized by autoradiography of an agarose gel on which the RT-PCR products have been electrophoretically separated. Indicated are the progesterone receptor product and the internal standard, RPL19, as well as the hormonal treatments in vivo. Uterus was used as a positive control and small intestine as a negative control.*



This raised the question whether the LH-R-mediated decrease in apoptosis could be mediated via PR signalling. To address this issue, two different PR-antagonists, Org 31710 and RU 486, were used. Granulosa cells were isolated from rats treated *in vivo* either with eCG only or with additional treatment with hCG for 12 h. Cells were incubated with or without addition of PR-antagonists for 24 h in serum-free medium.

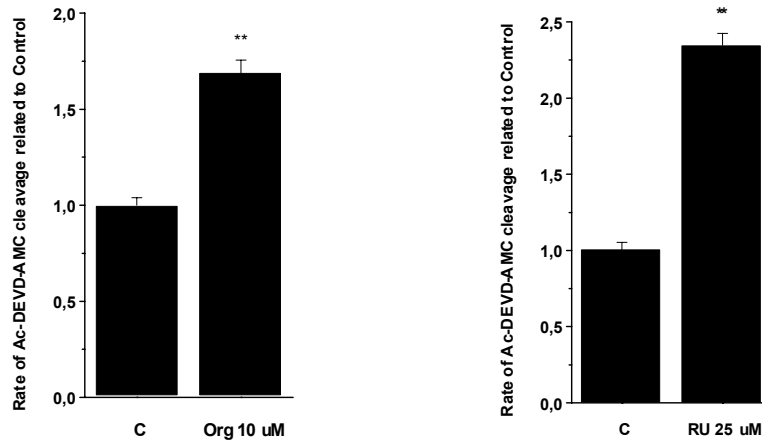
The degree of spontaneous development of internucleosomal DNA fragmentation *in vitro* was significantly reduced in granulosa cells isolated from rats treated with hCG, compared with granulosa cells isolated from rats only treated with eCG. The inhibitory effect on DNA fragmentation was dose-dependently reversed by addition of PR-antagonists to the culture medium during incubation.



**Figure 15.**

*Effect of the progesterone receptor antagonists Org 31710 and RU 486 on rat granulosa cell DNA fragmentation in vitro. Antagonists were added to the culture medium during 24 h incubation of granulosa cells isolated from 48 h eCG-primed animals with or without (Control) additional treatment with hCG for 12 h. Fluorospectrophotometric measurements showed a dose-dependent reversion by Org 31710 as well as by RU 486 of the inhibition of DNA fragmentation in vitro induced by hCG treatment in vivo. In the figure showing the experiments with Org 31710, n=6-18 (\*\* : P<0.01 compared to Org 0 µM). In the figure showing the experiments with RU 486, n=3 (\* : P<0.05, \*\*: P<0.01, compared to RU 0 µM).*

In addition to internucleosomal DNA fragmentation, caspase-3 activity, another event in the apoptotic cascade, was studied. Addition of Org 31710 or RU 486 to the culture medium caused a significant increase in caspase-3 activity compared to controls.



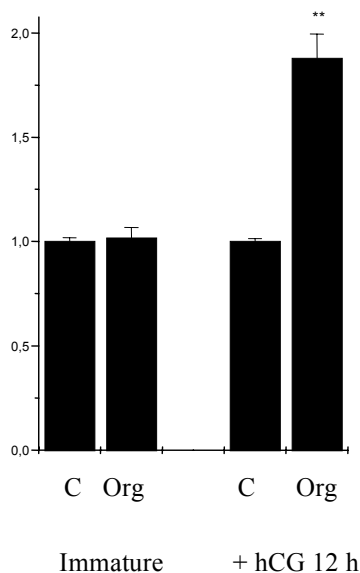
**Figure 16.**

Caspase-3 activity was measured in granulosa cells isolated from hCG-treated rats after incubation in serum-free medium with or without addition of Org 31710 (10  $\mu$ M) or RU 486 (25  $\mu$ M). The fluorogenic substrate used was Ac-DEVD-AMC (\*\* :  $P < 0.01$ ) ( $n=3$ ).

DNA fragmentation and caspase-3 activity was also studied after *in vitro* treatment with PR-antagonists of granulosa cells not exposed to LH receptor stimulation *in vivo*. Neither DNA fragmentation nor caspase-3 activity was affected in these cells, not expressing functional PR.

In addition, experiments using granulosa cells isolated from immature rats, not treated with any hormone, showed no effect on DNA fragmentation after addition of Org 31710 to the culture medium, compared to control.

Apoptotic Index



**Figure 17.**

Effect of the progesterone receptor antagonist Org 31710 on rat granulosa cell DNA fragmentation *in vitro*. Org 31710 (10  $\mu$ M) was added to the culture medium during incubation of granulosa cells isolated from immature rats and from rats treated with eCG for 48 h, followed by hCG-treatment for 12 h. Fluorospectrophotometric measurements showed a significant increase in Apoptotic Index, reflecting DNA fragmentation, in granulosa cells isolated from hCG-treated rats, but not in granulosa cells isolated from immature rats.  $n=9$ . (\*\* :  $P < 0.01$  compared to Control).

Since progesterone has been reported to mediate effects in granulosa cells not expressing PR via a GABA receptor-like receptor (Peluso and Pappalardo 1998), we wanted to see if this signalling pathway could be operating in the luteinizing preovulatory granulosa cells used in our experiments. However, addition of two different GABA receptor-antagonists (picrotoxin and bicuculline) as well as of a GABA agonist (muscimol) to the culture medium did not affect the degree of internucleosomal DNA fragmentation in these cells.

Since the progesterone receptor acts as a transcription factor, solid reasons exist to assume that it may regulate genes of importance for apoptosis development in granulosa cells. The microarray technique offers the possibility to compare the expression pattern of several thousands of genes simultaneously. Using this technique, we have studied the gene expression in rat luteinizing granulosa cells isolated from preovulatory follicles, exposed to hCG for 12 h *in vivo* and incubated for 24 h in the presence or absence of the progesterone receptor antagonist Org 31710 (10  $\mu$ M). This generates a model to study not only progesterone receptor-mediated effects on apoptosis, but also other effects of progesterone during the process of luteinization.

A preliminary analysis shows that a total of 83 genes were up- or down-regulated (43 and 40 respectively) more than 1.8-fold in duplicate samples hybridized to Affymetrix's rat microarray chips. The chip contains 8.800 genes, out of which approximately 3.400 were classified as present in our control RNA-pool.

In summary, these results suggest that LH receptor-mediated differentiation *in vivo* makes rat granulosa cells less susceptible to apoptosis. This effect seems to be, at least partly, mediated by the progesterone receptor.

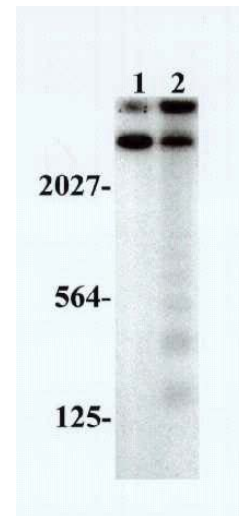
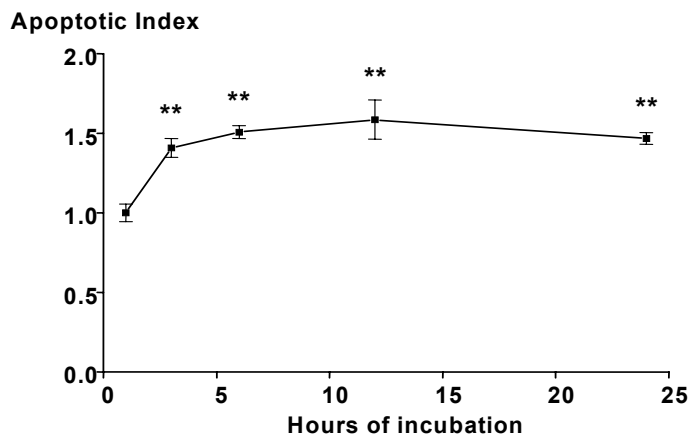
# Progesterone receptor-mediated inhibition of apoptosis in luteinizing human preovulatory granulosa cells

## (Paper III)

To study the effect of *in vivo* differentiation on spontaneous apoptosis development in human granulosa cells, luteinizing granulosa cells were collected from women in connection with ovum retrieval for *in vitro* fertilization (IVF). The cells were incubated with or without drug in serum-free medium.

Before follicular puncture, the women had been treated with a GnRH-agonist to down-regulate the endogenous gonadotropin stimulation of follicular growth, followed by rFSH to induce a cohort of follicles to grow simultaneously, and finally, when the follicles had reached a satisfactory size (as determined by ultrasonography), an ovulation-inducing dose of hCG.

Initially, the spontaneous development of apoptosis in luteinizing granulosa cells during 24 h incubation in serum-free medium was studied. Spontaneous internucleosomal DNA fragmentation in granulosa cells isolated from rats treated with PMSG *in vivo* and cultured for 24 h in serum-free medium is markedly increased in a time-dependent manner with a linear increase in the degree of internucleosomal DNA fragmentation over 24 h (Tilly *et al.* 1992). The increase in internucleosomal DNA fragmentation during the same time period in human cells, which *in vivo* had been subjected to LH receptor stimulation, was only 1.5-fold, a significant increase observed after 3 h, with no further increase observed.

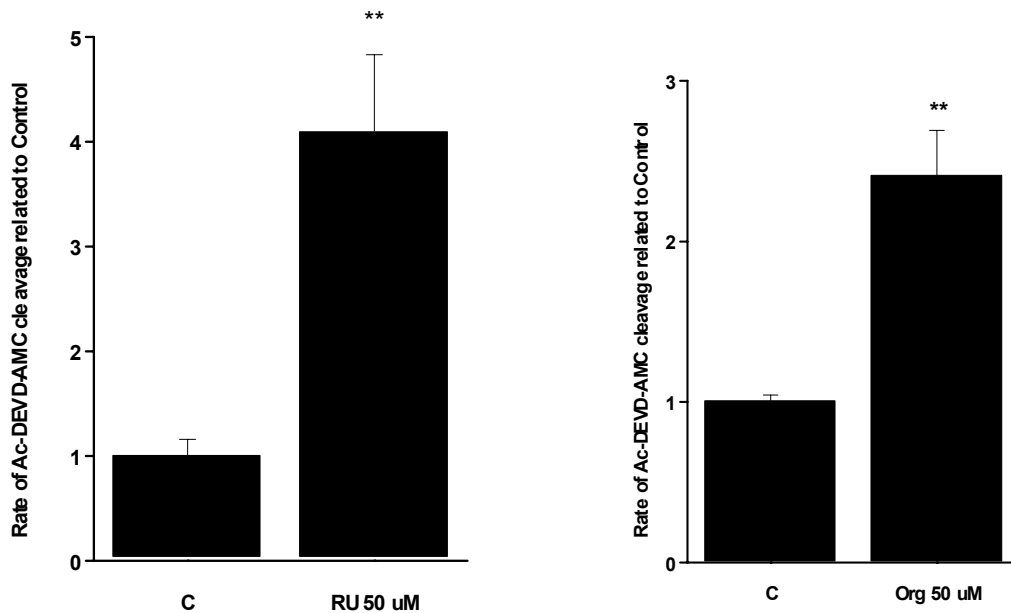


**Figure 18.**

Spontaneous apoptosis development in cultured human luteinizing granulosa cells after incubation in serum-free medium for various periods of time. The degree of internucleosomal DNA fragmentation was quantified fluorospectrophotometrically and visualized by gel electrophoretic separation of the <sup>35</sup>S-dATP-labelled DNA (lane 1 represents 0 h, lane 2 represents 24 h). An increase in internucleosomal DNA fragmentation was seen after 3h, with no further increase observed (\*\*:  $P < 0.01$  compared to 1 h).  $n = 4$ .

Thus, the spontaneous development of apoptosis in human luteinizing preovulatory granulosa cells was discrete.

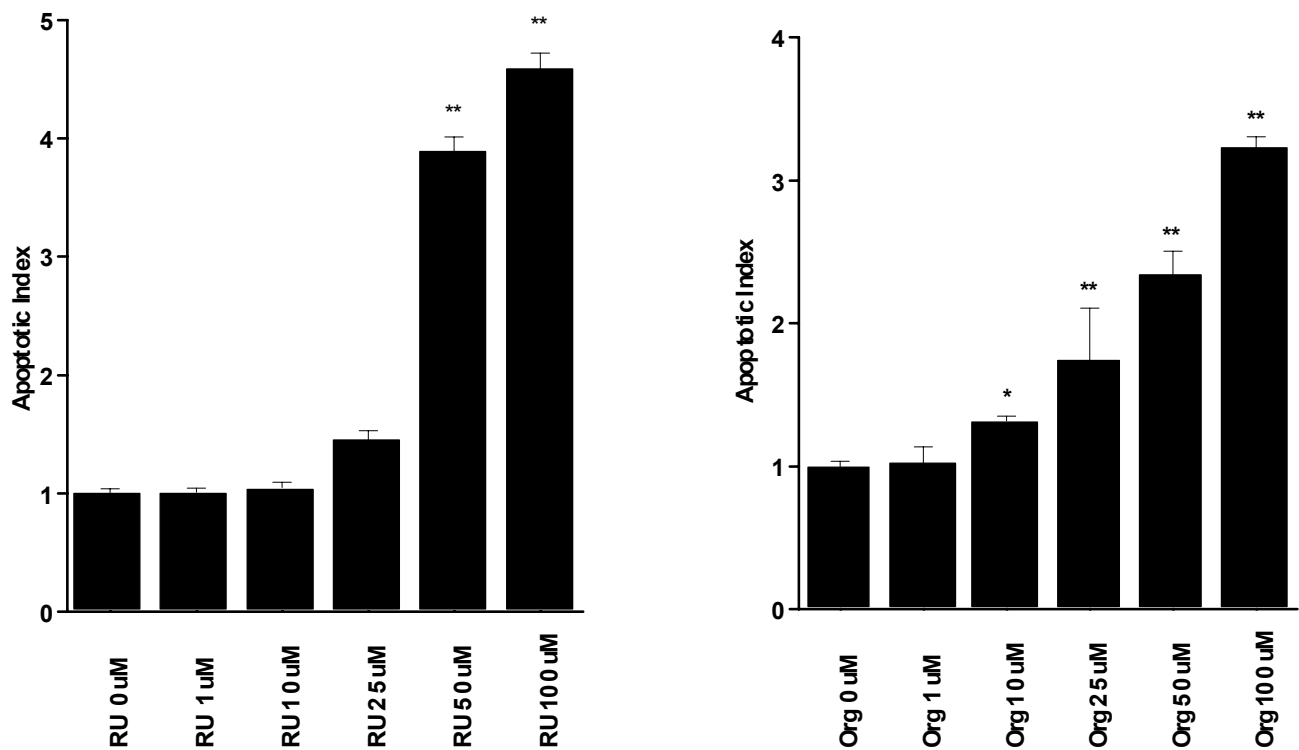
The effects of PR signalling in human luteinizing granulosa cells were investigated using the PR-antagonists Org 31710 and RU 486. As in the rat preovulatory luteinizing granulosa cells, the addition of antiprogestins during incubation for 24 h in serum-free medium, caused an increase in caspase-3 activity as well as a dose-dependent increase in internucleosomal DNA fragmentation. The effect of RU 486 was more pronounced than the effect of Org 31710.



**Figure 19.**

*Caspase-3 activity in cultured, antiprogestin-treated human luteinizing granulosa cells.*

*Caspase-3 activity was quantified in luteinizing granulosa cells after 24 h incubation in serum-free medium, with or without addition of RU 486 (50  $\mu$ M) or Org 31710 (50  $\mu$ M). Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) was used as fluorogenic substrate. n=5-7 (\*\* : P < 0.01).*



**Figure 20.**

*Induction of apoptosis in human luteinizing granulosa cells in vitro by addition of the antiprogesterins RU 486 or Org 31710 to the culture medium. Addition of RU 486 (1µM-100 µM) to the culture medium during 24 h incubation caused a dose-dependent increase in internucleosomal DNA fragmentation, quantified fluorospectrophotometrically and expressed as apoptotic index. (\*\* :  $P < 0.01$  compared to RU 0 µM).  $n = 9$ , except for 25 µM, where  $n = 3$ .*

*Addition of Org 31710 (1 µM – 100 µM) to the culture medium during 24 h incubation also caused a dose-dependent increase in internucleosomal DNA fragmentation, quantified fluorospectrophotometrically. (\*\* :  $P < 0.01$ , \* :  $P < 0.05$  compared to Org 0 µM).  $n = 3-9$ .*

Org 31710 is a highly selective PR-antagonist with no other known hormonal interactions except for weak interactions with the androgen receptor (Hurd *et al.* 1997, Kloosterboer *et al.* 1994,). In contrast, RU 486, in addition to its antiprogesterin activity, has antiglucocorticoid activity and also binds to the androgen receptor (Kloosterboer *et al.* 1994, Sánchez-Criado *et al.* 1993). To study the possible involvement of glucocorticoid or androgen receptor interaction with RU 486 in the luteinizing granulosa cells used in our experiments, dexamethasone or dihydrotestosterone in different doses was added to the culture medium. Neither of these drugs affected the degree of internucleosomal DNA fragmentation in human luteinizing preovulatory granulosa cells. Dexamethasone was also added in combination with different doses of RU 486, without affecting the apoptosis-inducing effect caused by RU 486.

No effect on internucleosomal DNA fragmentation was seen after addition of the GABA receptor-antagonist picrotoxin to the culture medium.

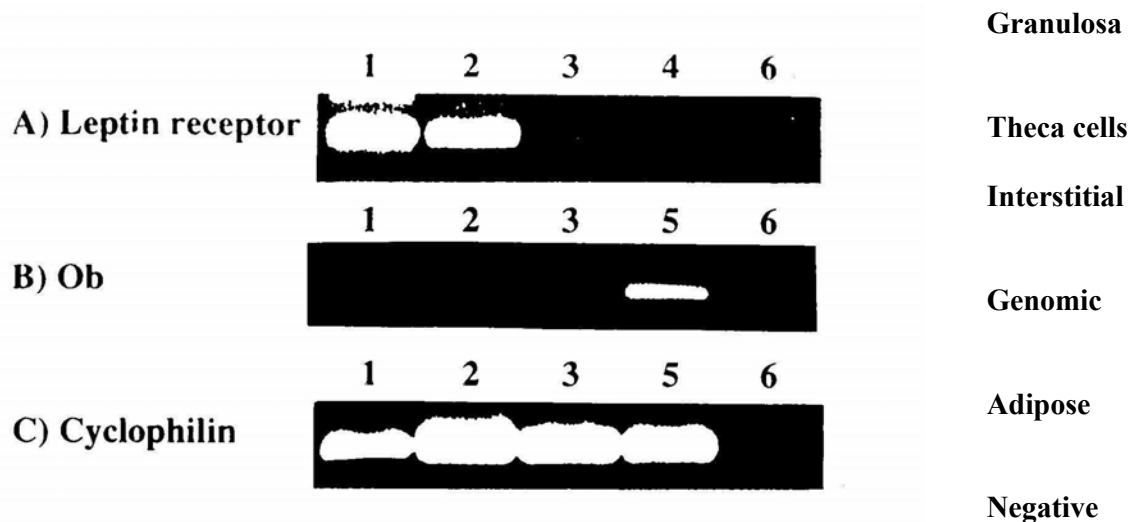
In conclusion, spontaneous development of apoptosis during incubation in serum-free medium is discrete in human luteinizing granulosa cells. The nuclear progesterone receptor is involved in regulating human luteinizing granulosa cell susceptibility to apoptosis after LH receptor stimulation *in vivo*.

## Leptin as a modulator of luteinizing granulosa cell steroidogenic activity

### (Paper IV)

To investigate whether leptin could exert any direct effects on the human ovary which might be involved in regulating luteinizing granulosa cell survival, we first studied the expression of leptin receptors and of leptin in ovarian follicular cells. Ovarian and adipose tissue were obtained from women with regular menstrual cycles undergoing laparotomy for reasons unrelated to ovarian pathology.

Using RNase protection assay (RPA), we were able to detect mRNA expression of a short isoform of the leptin receptor, but not of the full-length receptor. As the sensitivity of this assay might be too low to detect a low-expressed mRNA, reverse transcriptase polymerase chain reaction (RT-PCR) was performed. Using this method, long leptin receptor transcripts were detected in granulosa cells, theca cells and interstitial cells (DNA sequencing verified the identity of the cDNA). No expression of leptin mRNA was found in any of these cell types.



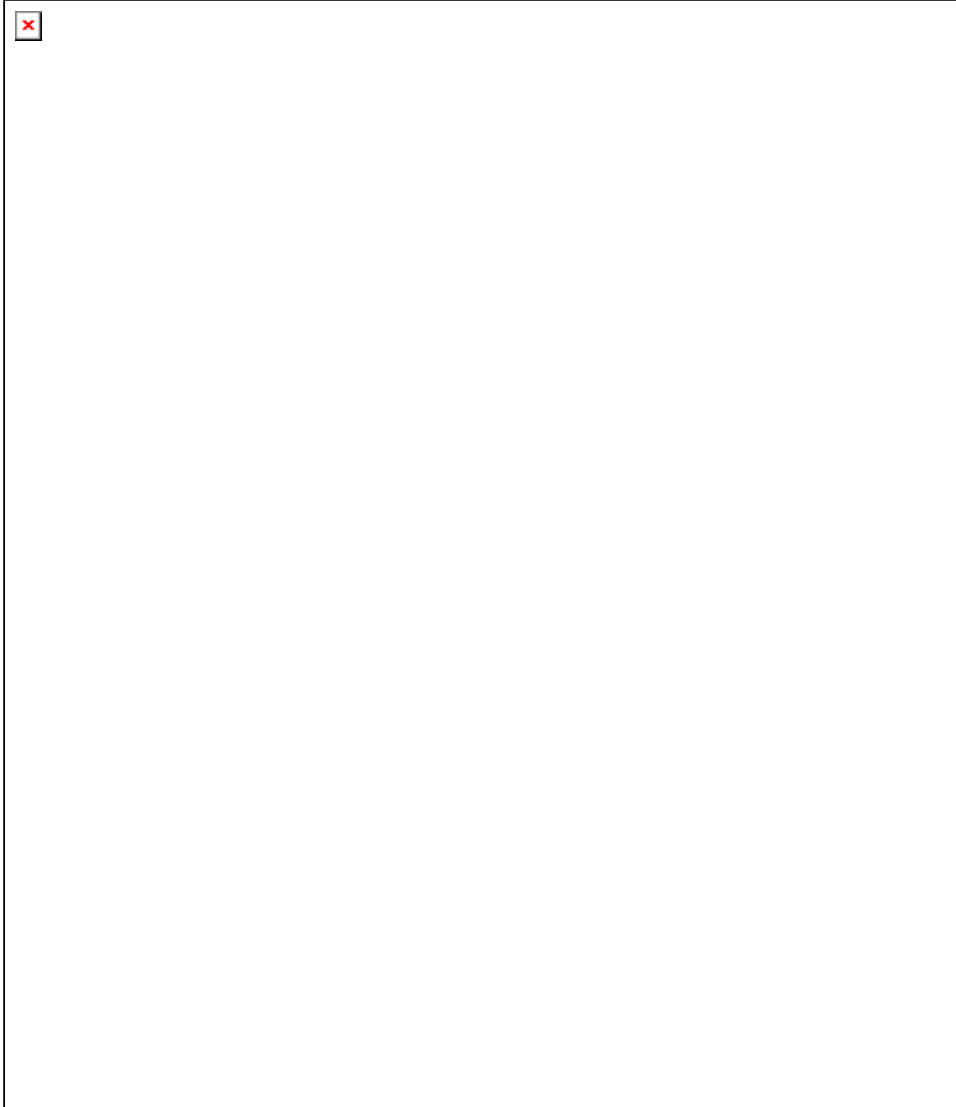
**Figure 21.**

*Tissue distribution of mRNA encoding the long isoform of the leptin receptor (A), ob (=leptin) (B) and cyclophilin (C), analysed by RT-PCR.*

Another finding, speaking against local production of leptin in the ovary was that levels of leptin in follicular fluid were equivalent to levels found in serum in four patients examined.

An intriguing question was whether leptin was capable of inducing a biological response in luteinizing human granulosa cells. Luteinizing granulosa cells, obtained from women under IVF-treatment were cultured for various periods of time in a medium containing testosterone as aromatase substrate and with or without addition of LH (to stimulate estradiol production) and /or leptin. Before addition of hormones to the cell cultures, the cells were precultured for 2-4 days, to down-regulate the high endogenous activity of gonadotropins.

Leptin had no effect on basal estradiol production, but decreased the LH-stimulated estradiol production.



**Figure 22.**  
*Effects of LH (0.1 ng/ml) and leptin (100 ng/ml) on estradiol production in primary cultures of human luteinizing granulosa cells during culture days 2-4 (A) and 4-6 (B). Data are presented as the mean  $\pm$  SEM. (\*\* :  $P < 0.01$ ).  $n = 4$ .*

The presented data show that leptin modulates steroidogenesis *in vitro* in human luteinizing granulosa cells. Leptin is suggested to act in an endocrine manner, since mRNA for leptin receptors, but not for leptin, was found in the ovarian follicular cells.



# CONCLUSIONS

- I Cell-concentration-dependent apoptosis is an important factor to take into consideration when interpreting in vitro data. Oxidants/free radicals seem to be involved in cell-concentration-dependent apoptosis in cultured rat granulosa cells.  
(Paper I)
  
- II LH receptor-mediated differentiation of preovulatory human and rat luteinizing granulosa cells, makes these cells less susceptible to apoptosis compared to granulosa cells at earlier stages of differentiation.  
(Paper II and Paper III)
  
- III The LH receptor-mediated decrease in apoptosis susceptibility in luteinizing human and rat granulosa cells seems to be partly mediated by expression and stimulation of the nuclear progesterone receptor.  
(Paper II and Paper III)
  
- IV Leptin can modulate steroidogenesis in human luteinizing granulosa cells, indicating an important direct role of leptin in endocrine regulation of cellular events of importance for reproductive success.  
(Paper IV)



# DISCUSSION

The question what mechanisms regulate life and death is probably as old as mankind itself. In this thesis we have tried to elucidate some of the events involved in nature's intricate regulation of the survival of the species, by studying factors that regulate apoptosis in the ovary.

We chose to work with a very fine-tuned event, elicited by the ovulatory LH-surge, namely the conversion of the preovulatory follicle to become a luteinizing follicle. At all other stages during follicular development, the incidence of apoptosis is very high, with only a minute fraction of follicles surviving to the next developmental stage. However, in the preovulatory follicle, exposed to the surge of LH, the susceptibility to undergo apoptosis seem to be dramatically changed, as the number of corpora lutea roughly equals the number of preovulatory follicles.

Since luteinizing granulosa cells are characterized by the expression of progesterone receptors as well as a high steroidogenic activity, it was pertinent to study factors involved in progesterone receptor signalling as well as factors involved in regulation of granulosa cell steroidogenic activity.

## OXIDATIVE STRESS

### Generation of free radicals / reactive oxygen species

The generation of oxidative free radicals / reactive oxygen species (ROS) (oxidative stress) is a consequence of normal cellular respiratory metabolism and reduction-oxidation (redox) reactions. In most eukaryotes, mitochondria are the major source of free-radical reactive oxygen species. Other sites include the microsomal cytochrome P450, plasma membrane NAD/NADPH systems and peroxisomes (Andersson *et al.* 1999). Endogenous eukaryotic cellular mechanisms for reducing oxidative stress include the enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase, reduced glutathione (GSH) and thioredoxin.

### Cell-concentration-dependent apoptosis

In this thesis, we have shown that cell concentration is an important factor to take into consideration when interpreting *in vitro* data, concerning both apoptosis and other parameters. Data in this thesis show that the higher the concentration of cells during incubation, the more pronounced is the spontaneous development of apoptosis. We suggest that part of this cell-concentration-dependent apoptosis may be due to the action of oxidants, since addition of scavengers of oxidants to the culture medium decreased the spontaneous development of apoptosis. We were not able to demonstrate indirectly the existence of oxidants in our culture medium but it is a well-known fact that oxidants are highly reactive compounds with only transient existence (Ischiropoulos 1998). The scavengers used were superoxide dismutase, scavenging superoxide ions (Laloraya *et al.* 1988) and 1,10-phenantroline, chelating stored iron ( $\text{Fe}^{3+}$ ) or reduced iron ( $\text{Fe}^{2+}$ ), thereby protecting cells from hydroxyl radicals produced

from hydrogen peroxide via the Fenton reaction (Hiraishi *et al.* 1993). Heparin is a multifunctional glucosaminoglycan, which has been reported to act as a scavenger of free radicals, possibly by binding and sequestering ions like  $\text{Fe}^{2+}$ , thereby preventing the Fenton reaction, or alternatively acting as a free radical sink (Albertini *et al.* 1996, Hiebert and Ping 1997, Maeda *et al.* 1993, Ross *et al.* 1992). It was recently demonstrated that heparin can inhibit apoptosis by interacting with activator protein 1 (AP-1), a crucial mediator for oxidant-induced apoptosis (Ishikawa and Kitamura 1999).

ROS production has been suggested to contribute to the induction and/or activation of matrix metalloproteinases (MMPs) (Morita-Fujimura *et al.* 2000, Wenk *et al.* 1999). 1,10-phenantroline is, in addition to its scavenger function, an inhibitor of  $\text{Zn}^{2+}$ -dependent metalloproteases (Borrego *et al.* 1994). This raises the possibility that part of the apoptosis-inhibiting effect of 1,10-phenantroline noted in Paper I, may be due to modulated MMP-activity. Several MMPs have been shown to be expressed in the mouse ovary. Stromelysin-3 was found to be one of the most abundantly expressed MMPs and, interestingly, the mRNA expression of this MMP has been found in areas where extensive apoptosis occurs (Hägglund *et al.* 1999).

### **Targets for oxidant actions**

The literature suggests several different targets for oxidant actions in cells. ROS can alter the redox potential of proteins by a variety of means, e.g. covalent modification of sulphhydryl groups, a major mechanism for regulating protein function. Binding of proteins to other macromolecules (e.g. transcription factors to DNA), altered activity of enzymes like protein kinases and phosphatases and of transcription factors requiring critical cysteine residues and altered concentration of cytosol  $\text{Ca}^{2+}$ , offer many sites for modulating cellular metabolism.

Oxidative damage and apoptosis share many features: membrane blebbing, cytoskeletal disruption, changes in membrane lipids including movement of phosphatidyl serine to the plasma membrane surface, decreased  $\text{NAD}^+$ , ATP and mitochondrial membrane potential, increased  $\text{Ca}^{2+}$ , ADP-ribosyl-transferase, DNA damage and synthesis of heat shock proteins (HSP), glutamyl-S-transferase (GST), c-fos and c-myc and  $\text{Ca}^{2+}$  protease expression/activity (Buttke and Sandstrom 1994, Gius *et al.* 1999, Payne *et al.* 1995).

Another theory concerning the involvement of ROS in apoptosis is that cytochrome c, which is localized to the outer surface of the inner mitochondrial membrane, can oxidize superoxide ions back to  $\text{O}_2$  and then be oxidized itself by cytochrome oxidase. If the production of superoxide ions exceeds the capacity of cytochrome c, an apoptotic mechanism is thought to start, resulting in release of cytochrome c to the cytoplasm, with subsequent activation of the caspase pathway of apoptosis (Skulachev 1998).

### **Ovarian protection against oxidative stress**

In the ovary, it was recently suggested that the ability of gonadotropins to prevent apoptosis in rat ovarian follicles was linked to a shift in the bcl-2/bax/bcl-x-rheostat with dominance of apoptosis inhibition, primarily by decrease in bax and constitutive bcl-2 and bcl-x-mRNA-levels (Tilly *et al.* 1995). In the rat, SOD as well as catalase increase during follicular development and are under the influence of gonadotropins (Laloraya *et al.* 1988, Peterson and Stevenson 1992). SOD-mRNA in the rat corpus luteum is induced by prolactin and placental lactogens and is suggested to play an important role in maintaining luteal cell

integrity and steroidogenic activity (Sugino *et al.* 1998). In the human, stimulation of luteal Cu-Zn-SOD expression by hCG has been suggested to be important in maintaining luteal cell integrity when pregnancy occurs (Sugino *et al.* 2000). Immunohistochemical studies, using normal cycling human ovaries, suggest that the expression of Mn-SOD and Cu-Zn-SOD closely correlates with steroidogenesis in the human ovary (Suzuki 1999).

Cu-Zn-SOD-deficient female mice are subfertile. FSH and LH levels in these mice are suppressed and have been suggested to be the primal defect causing the subfertility (similar numbers of eggs are produced in Cu-Zn-SOD-deficient mice and littermate controls upon pharmacological superovulation). However, a local elevation of superoxide radicals within the ovary, affecting general steroid biosynthesis, cannot be ruled out (Matzuk *et al.* 1998). Interestingly, suppression of intracellular superoxide dismutase activity by antisense oligonucleotides causes inhibition of progesterone production by rat luteal cells (Sugino *et al.* 1999). In the rabbit corpus luteum, endogenous Mn-SOD protein was shown to acutely down-regulate Bax expression (Dharmarajan *et al.* 1999).

## **PROGESTERONE SIGNALLING**

### **The nuclear progesterone receptor**

Data presented in this thesis show that stimulation of progesterone receptors is involved in the regulation of survival of rat and human luteinizing granulosa cells. These cells have *in vivo* been subjected to stimulation of the LH receptor with concomitant expression of nuclear PR. Indeed, the classical way of progesterone to mediate effects is via the nuclear progesterone receptor. However, in addition to the nuclear PR, several other ways of progesterone mediating effects have been demonstrated;

### **The GABA receptor**

Several studies in various species have shown the importance of progesterone in regulating cell survival (Pecci *et al.* 1997, Peluso and Pappalardo 1994, Peluso and Pappalardo 1998, Peluso and Pappalardo 1999, Rueda *et al.* 2000). In immature and preovulatory (but not LH receptor-stimulated) rat granulosa cells, progesterone has been attributed an apoptosis-inhibiting effect, though not mediated by the nuclear PR, but by a GABA receptor-like receptor (Peluso and Pappalardo 1998). The existence of GABA receptors in the ovary has indeed been reported (Erdö and Lapis 1982, Schaeffer and Hsueh 1982), though ovarian effects mediated by progesterone using classic GABA receptors have not been reported. However, in the brain, progesterone signalling via GABA receptors is well studied (Brann *et al.* 1995, MacDonald *et al.* 1991). To address the question whether the GABA receptor signalling pathway was activated by progesterone in our experiments, GABA receptor antagonists (picrotoxin and bicuculline) as well as the GABA agonist muscimol were added to the cultures. However, none of these drugs influenced the degree of DNA fragmentation in luteinizing human or rat granulosa cells during 24 h incubation in serum-free medium.

## **The glucocorticoid receptor**

Another receptor by which progesterone may mediate effects, is the glucocorticoid receptor in the rat corpus luteum (lacking PR) (Sugino *et al.* 1997). Progesterone binding to a membrane-bound protein in immature bovine granulosa cells has also been reported (Rae *et al.* 1998). In addition, an orphan nuclear receptor, activated by pregnenolone and progesterone, was recently characterized in mouse liver, intestine, kidney and stomach (Kliwer *et al.* 1998). Thus, in cells not expressing the nuclear PR, progesterone may mediate effects using other signalling pathways. To investigate whether progesterone exerted any effects via glucocorticoid receptors in our experiments, dexamethasone was added to the cultures during incubation. However, no effect was seen on DNA fragmentation after addition of dexamethasone, excluding the possibility of progesterone signalling via the glucocorticoid receptor in human or rat luteinizing granulosa cells to regulate apoptosis *in vitro*.

## **Non-receptor-mediated effects**

In developing follicles, progesterone has been shown to inhibit FSH-stimulated follicular maturation (Goodman and Hodgen 1982), steroidogenesis (Schreiber *et al.* 1980) and LH receptor expression (Schreiber *et al.* 1982, Skinner 1999). Negative feedback actions of progesterone on gonadotropin-releasing hormone secretion have recently been shown to be transduced by the classical progesterone receptor (Skinner 1999). However, there are also reports that progesterone can exert stimulatory effects on follicular recruitment and development, particularly when serum LH is suppressed (Richards 1982). Interestingly, no classical nuclear progesterone receptors are found in granulosa cells in developing follicles until the LH-surge. Thus, effects of progesterone in these cells may be mediated by interaction with other receptors. It has been suggested that a receptor-mediated process may not be the only means by which progesterone acts, but it may also act on any element of the process affecting its own biosynthesis (Rothchild 1996).

In this thesis, the role of progesterone in the preovulatory, LH-R-stimulated follicle, expressing the nuclear progesterone receptor in the granulosa cells, is discussed.

## **PROGESTERONE AND APOPTOSIS**

Almost all ovarian follicles undergo atresia during follicular development (Billig *et al.* 1996, Hsueh *et al.* 1994, Kaipia and Hsueh 1997). This hormonally controlled apoptotic process occurs throughout follicle development, with an extensive reduction in the number of follicles present at birth (Gougeon 1996). This drastic reduction is, however, not present among preovulatory follicles responding to the ovulatory LH-surge, as the number of corpora lutea roughly equals the number of preovulatory follicles.

In this thesis, we demonstrate that LH receptor-mediated differentiation of granulosa cells *in vivo* is important for making granulosa cells in the preovulatory follicle less susceptible to apoptosis. This effect seems to be at least partly, mediated by signalling via the PR, since treatment of the luteinizing granulosa cells *in vitro* with the progesterone receptor antagonists Org 31710 or RU 486 causes an increase in internucleosomal DNA fragmentation as well as in caspase activity. No effects on internucleosomal DNA fragmentation or caspase activity were seen when granulosa cells isolated from rats not treated with hCG were

incubated with the same drugs. Indeed, this data was in part confirmed recently by Makrigiannakis with colleagues (Makrigiannakis *et al.* 2000).

RU 486 is widely used to study progesterone-mediated effects both *in vivo* and *in vitro* (DiMattina *et al.* 1986, DiMattina *et al.* 1987, Heikinheimo *et al.* 1995). This drug is also in clinical use for various purposes, including voluntary early pregnancy termination and emergency postcoital contraception (Cadepond *et al.* 1997). Treatment with RU 486 will influence endometrial development and function sufficiently to prevent implantation (Bygdeman *et al.* 1997, Task force on postovulatory methods of fertility regulation 1999). The endometrium seems to be more sensitive to withdrawal of progesterone influence than are follicular development and ovulation. Higher doses of RU 486 will also inhibit ovulation (Bygdeman *et al.* 1997).

One drawback of RU 486, concerning studies focusing on progesterone actions, is its well-known interaction with the glucocorticoid receptor (Kloosterboer *et al.* 1994), sometimes making it difficult to interpret data. In addition to interaction with the glucocorticoid receptor, a weak interaction with the androgen receptor has also been found (Sánchez-Criado 1993). No direct interactions with the estrogen receptor have been reported.

Org 31710 is a much more selective antiprogestin, with no other known hormonal interactions than a weak binding to the androgen receptor (Hurd *et al.* 1997, Kloosterboer *et al.* 1994).

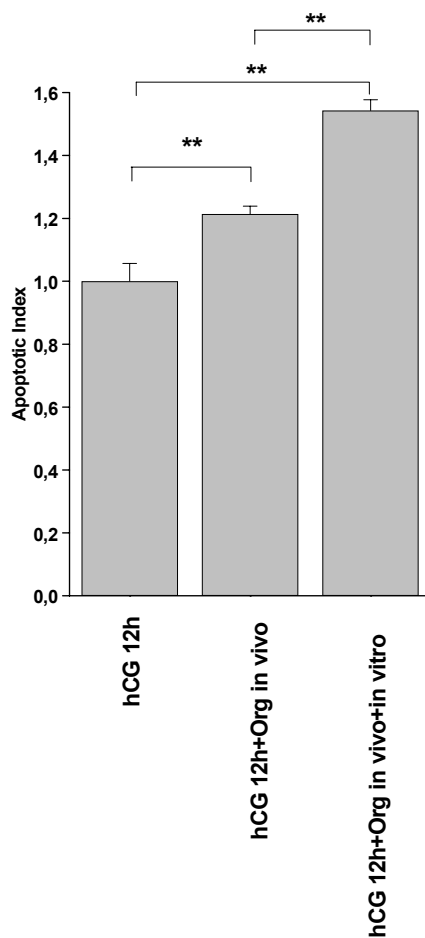
Org 31710 and RU 486 both show a higher relative binding affinity to PR-A than does progesterone (Schoonen *et al.* 1998). RU 486 as well as Org 31710 bind to the PR with subsequent binding of the complex to PREs (progesterone responsive elements). RU 486 promotes a stronger interaction of PR with PREs *in vitro* than does the synthetic progestin R5020 (Gass *et al.* 1998). This may explain the inability of exogenously added progesterone in our experiments to reverse the effects of the antiprogestins.

The effect of RU 486 on internucleosomal DNA fragmentation as well as caspase activity in our experiments was more accentuated than the effect of Org 31710 at the same doses. To address the question whether RU 486 interacted with glucocorticoid and/or androgen receptors in the granulosa cells, dexamethasone or dihydrotestosterone (DHT) was added to the culture medium. Neither of these drugs affected the degree of internucleosomal DNA fragmentation in luteinizing human granulosa cells, excluding the possibility that the apoptotic effect of RU 486 could be mediated by the glucocorticoid or the androgen receptor.

The more pronounced effect mediated by RU 486 may be due to different PR interactions, and even differences in subsequent interaction of the PR-PR-antagonist complexes with PREs. Another possibility is the interaction of RU 486 with yet unidentified targets in these cells. Interestingly, RU 486 has been shown to interfere with steroidogenesis in cultured luteinizing granulosa cells by enzymatic inhibition (DiMattina *et al.* 1986, DiMattina *et al.* 1987).

Addition of progesterone to the culture medium of luteinizing granulosa cells could not accentuate the apoptosis inhibition already established in these LH receptor-stimulated cells. Characteristic of luteinizing granulosa cells is their endogenous, high production of progesterone. Thus, the PR expressed may already be saturated by the endogenous levels of progesterone. Experiments using the progesterone synthesis inhibitor aminogluthetamide (inhibiting P450<sub>scc</sub>) in our laboratory, showed a decrease in the amount of accumulated progesterone in spent medium by 90-95 %. However, the degree of DNA fragmentation in these cells after 24 h incubation was not affected compared to controls. As the production site and ligand binding site (PR) are in such close proximity in the granulosa cells, the remaining amount of progesterone may be sufficient to saturate the PR. Another important point to take into consideration is that the luteinizing granulosa cells already express PR at the time of isolation and have been exposed to high levels of progesterone for several hours *in vivo*.

Indeed, we have performed *in vivo* experiments in which immature rats were treated with or without Org 31710 in addition to hCG-treatment. A significant increase in DNA fragmentation could be detected in luteinizing granulosa cells after treatment with Org 31710 *in vivo* with subsequent incubation for 24 h. This apoptosis-inducing effect was further accentuated if the luteinizing granulosa cells exposed to Org 31710 *in vivo* were also exposed to Org 31710 *in vitro* during 24 h culture (Figure 23).



**Figure 23.** Effect of Org 31710 on rat granulosa cell apoptosis *in vitro* after exposure to the drug *in vivo* with or without subsequent exposure to the drug *in vitro*. Rats were treated with hCG (50 IU) *in vivo* for 12h. One group were pretreated with Org 31710 (1 mg/kg) one hour before the start of hCG-treatment. Granulosa cells were isolated and incubated with or without addition of Org 31710 (10  $\mu$ M) to the culture medium. DNA-fragmentation was measured fluorospectrophotometrically after 24 h of incubation and expressed as apoptotic index. (\*\*;  $P < 0,01$ ).  $n = 6$ .



A possible way to elucidate the role of PR-mediated effects in granulosa cell apoptosis *in vivo* is to pharmacologically block the increase in LH receptor stimulation, concomitant with either the endogenous LH-surge or exogenously added hCG. For this purpose, we used the drug pentobarbitone sodium (Nembutal), which blocks the preovulatory surge of LH (Braw and Tsafiriri 1980, Everett and Sawyer 1950). Unfortunately, data from these experiments were not conclusive, since some of the treated animals showed signs of ovulation, indicating that the LH-surge was not completely blocked.

## **DEVELOPMENTAL CHANGES IN INCIDENCE OF APOPTOSIS**

Differentiation of granulosa cells within an ovarian follicle is a prerequisite for ovulation to occur. During the development of the ovarian follicle from the primordial to the preovulatory stage, the vast majority of follicles will degenerate. One developmental stage of fundamental importance is when the antral follicle, starting to express FSH receptors, becomes dependent on FSH for survival. The antral follicles expressing FSH receptors, but not being exposed to sufficient amounts of FSH, will degenerate (Chun *et al.* 1996, Hsueh *et al.* 1994).

The follicle which survives will during its further differentiation start to express LH receptors. The expression of LH receptors is a prerequisite for LH to trigger ovulation of the follicle. In connection with the ovulatory LH-surge, a transient upregulation of progesterone receptors within the granulosa cells occurs.

Data presented in this thesis show that stimulation of progesterone receptors is involved in regulating the survival of rat as well as of human luteinizing granulosa cells. The highest doses of RU 486 used showed a more pronounced degree of apoptosis in luteinizing granulosa cells than was found in non-LH receptor-stimulated granulosa cells exposed to RU 486. This indicates that granulosa cells expressing PR seem to be more sensitive to progesterone withdrawal than granulosa cells not expressing PR. This is in line with FSH-dependence of FSH receptor-expressing granulosa cells in developing follicles. Indeed, in the progesterone receptor knockout mouse, the follicles do not ovulate but persist, giving the ovaries a polycystic appearance (Lydon *et al.* 1995).

## **PR TARGET GENES**

The progesterone receptor acts as a transcription factor upon ligand activation. To understand the downstream effects elicited by this event, identification of PR target genes is crucial. A few data concerning target genes for PR action are available. The proteases ADAMTS-1 (A disintegrin and metalloproteinase with thrombospondin-like motifs) and cathepsin L (a lysosomal cystein protease) are transcriptional targets for progesterone receptor action and are suggested to play critical roles in ovarian follicular rupture (Robker *et al.* 2000). Human insulin receptor substrate-2 (IRS-2) has also been identified as a primary progesterone responsive gene, indicating a mechanism by which progesterone can modulate the effects of insulin, IGF-I and cytokines on cell proliferation, differentiation and homeostasis (Vaßen *et al.* 1999). Targeted deletion of the PR gene in mice (PRKO) causes a mouse phenotype with numerous reproductive abnormalities (Lydon *et al.* 1995). One of the defects in PRKO mice is failure to ovulate (Lydon 1995). PRKO mice develop mature follicles responding to gonadotropins, but fail to ovulate, giving the ovaries a polycystic

appearance with viable oocytes enclosed by luteinized follicles (Lydon *et al.* 1995). It was recently shown that the expression of the two proteases ADAMTS-1 and cathepsin L was aberrant in PRKO compared to wild-type mice (Robker *et al.* 2000).

By selective ablation of PR-A in mice (PRAKO), it was shown that PR-B modulates a subset of reproductive functions of progesterone by regulation of a subset of progesterone-responsive target genes (Mulac-Jericevic *et al.*, 2000). These animals appear normal except that PRAKO females are infertile. They produce a reduced number of oocytes, but fail to implant the oocytes due to lack of decidualization of stromal cells.

Others have shown that progesterone receptor stimulation in rat granulosa cells at the time of ovulation is required for expression of the PACAP (pituitary adenylate cyclase activating polypeptide) gene (Ko *et al.* 1999) and the PACAP receptor PAC<sub>1</sub> (Ko and Park-Sarge 2000).

The microarray technique is a valuable tool in decoding the different cellular events elicited by transcription factors. Since we were interested in finding PR-induced apoptosis regulating genes, experiments were designed in our laboratory to compare two different groups of granulosa cell mRNA. Both groups were isolated from rats treated *in vivo* with eCG for 48 h, followed by hCG-stimulation for 12 h. The luteinizing granulosa cells were then incubated for 24 h with or without the addition of Org 31710. Isolating mRNA from these two groups gives us the possibility to see what genes are involved in the cellular response to blockage of progesterone receptors in luteinizing granulosa cells *in vitro*. A large number of genes were either up- or down-regulated after treatment with Org 31710. Preliminary analysis of the microarray data indicates that progesterone receptor stimulation may affect expression levels of genes involved in several metabolic and functional pathways, including cholesterol synthesis, angiogenesis, follicular rupture and stress responses. Solid reasons exist to assume that some of these genes may be involved in the regulation of susceptibility to apoptosis development in granulosa cells. Studies regarding this issue are currently in progress.

## ANTIPROGESTINS IN THE CLINIC

RU 486 has been used clinically for emergency postcoital contraception as well as for voluntary early pregnancy termination (Bygdeman *et al.* 1997, Cadepond *et al.* 1997, Task force on postovulatory methods of fertility regulation 1999). For these indications, the endometrium has been the primary target for RU 486 treatment. Daily administration of RU 486 for several months, for ovulation suppression, may in humans be associated with endometrial hyperplasia (Murphy *et al.* 1995). Monthly premenstrual (late luteal phase) administration of RU 486 has been reported as well. However, this regimen proved unsuccessful, because the failure rate was too high (20 %). RU 486 induced cycle irregularities with retardation of the next ovulation (Van Santen *et al.* 1987).

Results from a study in which Org 31710 was given once monthly (day 1, 28, 56 etc) as a complement to a progestagen-only pill to improve cycle control were recently published (van Heusden *et al.* 2000). However, this resulted in effects on the hypothalamic-pituitary-ovarian axis, which made the results difficult to interpret. To our knowledge, no studies where an antiprogesterin is administered only in the late follicular phase have hitherto been reported. Based on the findings presented in this thesis, such a regimen could possibly interfere with PR-expressing granulosa cells in the follicle destined to ovulate, affecting the survival of these cells, thereby affecting the success of ovulation.

In the light of the data presented in this thesis, it is tempting to speculate over the possibility of interfering with ovarian cancer development by use of antiprogesterins. Indeed,

the results from a phase II study of RU 486 in refractory ovarian cancer were recently published (Rocereto *et al.* 2000), showing that RU 486 had activity against ovarian cancer resistant to cisplatin and paclitaxel.

## LEPTIN AS A MODULATOR OF THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS

Chehab with coworkers were the first to demonstrate that leptin had an impact on the reproductive system, when they showed that the sterility defect in *ob/ob* female mice could be corrected by leptin administration (Chehab *et al.* 1996). Later studies have suggested leptin as a metabolic gate to trigger the onset of puberty when the energy reserves are adequate, although evidence that leptin is the primary metabolic signal for initiating the onset of puberty is controversial (Cunnungham *et al.* 1999).

The negative influence of insufficient nutrition on the hypothalamic-pituitary-gonadal axis is primarily realized through reduced pituitary LH release, a presumed reflection of decreased hypothalamic GnRH secretion (Hileman *et al.* 2000). It has been found by several laboratories that leptin levels fall during fasting or food restriction in conjunction with decreased levels of LH and that administration of leptin during food restriction, either intracerebroventricularly or intraperitoneally, restores LH release (Hileman *et al.* 2000). Anti-leptin serum given to normally fed rats significantly depresses the LH-surge (Kohsaka *et al.* 1999).

Leptin stimulates GnRH release from isolated hypothalamic explants and FSH and LH release from anterior pituitaries of adult male rats *in vitro*, and releases LH, but not FSH, *in vivo* (Yu *et al.* 1997). Collectively, these data suggest that leptin acts centrally to influence reproduction, even though the question of whether these actions are exerted directly on GnRH neurons or indirectly through interneural connections remains unanswered.

When the series of experiments described in Paper IV were performed, not much was known about peripheral actions of leptin. One intriguing question was whether leptin was able to modulate reproduction directly on the ovarian level. The demonstration of leptin receptors in follicular cells and the modulation by leptin of granulosa cell steroidogenesis *in vitro* suggests a direct effect of leptin on the human ovary. Our work indicates an endocrine, rather than an auto/paracrine, action on the ovary, as transcripts of the *ob* gene could not be detected in human ovarian cells. In contrast, a recent report demonstrated leptin expression in luteinized, hCG-exposed granulosa cells from individuals undergoing hormonal treatment prior to *in vitro* fertilization (Cioffi *et al.* 1997). However, the granulosa cells used for analyses in our work differ from the granulosa cells used by Cioffi *et al.*, in that our cells were collected from women with regular menstrual cycles, not exposed to high levels of hCG.

Today it is evident that, in addition to its central actions, leptin also mediates direct effects in several peripheral tissues. The majority of leptin receptors present in peripheral tissues are truncated and have less signalling capacity (Bjorbaek *et al.* 1997). However, the observation that addition of leptin generates profound biological responses in cultured hepatocytes, adipocytes, muscle cells, hemopoietic cells, adrenocortical cells, endothelial cells and pancreatic islet cells supports a direct peripheral action of leptin (Dallongeville *et al.* 1998).

The modulation of ovarian steroidogenesis in thecal cells (Agarwal *et al.* 1999, Spicer and Francisco 1998) as well as in granulosa cells (Barkan *et al.* 1999, Brannian *et al.* 1999, Spicer and Francisco 1997, Zachow and Magoffin 1997, Zachow *et al.* 1999) of different species has now been demonstrated in several papers. Whether the signalling of leptin in

granulosa cells is mediated by the low-expressed long isoform of the leptin receptor or via a more highly expressed truncated form remains to be determined.

The finding that leptin is able to attenuate the synergistic action of IGF-I and FSH on estradiol production by rat ovarian granulosa cells (Zachow and Magoffin 1997) raised the question whether high leptin levels may contribute to infertility in some women with polycystic ovary syndrome (PCO). Some authors reported that a substantial proportion of women with PCO had higher leptin levels than expected for their body mass index, free testosterone and insulin sensitivity, suggesting that abnormalities in leptin signalling to the reproductive system may be involved in certain cases of PCO (Brzechffa *et al.* 1996). However, contradictory findings were soon reported by several independent laboratories, providing evidence against the concept of elevated leptin levels in PCO patients compared to those in age- and weight-matched control subjects (Caro 1997, Laughlin *et al.* 1997, Mantzoros *et al.* 1997b, Rouru *et al.* 1997). Jacobs and Conway recently speculated that in women with PCO, insulin-stimulated leptin secretion could be limited by insulin resistance in adipocytes. An important component of the obesity of women with PCO is accumulation of visceral fat, which secretes less leptin than subcutaneous fat. The authors therefore postulated that lower than appropriate satiety signals permit further development of obesity. Increasing obesity would then result in progressively severe insulin resistance with eventual disturbance of reproductive and metabolic function. At the ovarian level, high leptin concentrations might impair ovarian function by reducing the response to gonadotropin stimulation (Jacobs and Conway 1999).

Finally, it can be postulated that both too low and too high levels of circulating leptin may be hazardous for the success of reproduction. Low levels of leptin may be perceived by the body as harmful and preclude reproduction through a number of adaptive changes mediated by the hypothalamic-pituitary axis. High levels, as in obesity, may instead interfere locally by inhibiting steroidogenesis at the ovarian level. Because leptin influences the effect of steroid production in granulosa cells and is also involved in infertility, the possibility remains that apoptosis of granulosa cells may be the result of ovarian exposure to high levels of leptin, inhibiting local progesterone production. Experiments addressing this interesting issue have been conducted in our laboratory but no conclusive data have yet been obtained because of lack of appropriate amounts of experimental materials.

If technically possible, it could be speculated that a local blockage of the leptin receptor in the ovary or a reduction of circulating leptin levels, could restore fertility in subfertile obese women.

## **CONCLUDING REMARKS**

Elucidation of the mechanisms regulating survival and death of ovarian granulosa cells could in the future imply possibilities of interfering with cellular mechanisms involved in pathological conditions such as ovarian infertility and cancer as well as in mechanisms applicable to contraception.

In a wider perspective, decoding apoptotic pathways would be of great importance in the search for treatments for a wide range of diseases caused by a disturbance in the apoptosis/proliferation equilibrium, e.g. neurodegenerative diseases, AIDS and various cancers.

In this thesis, we have provided insight into some important events regulating apoptosis in ovarian luteinizing granulosa cells. The applicability of these findings in treatments for the conditions stated above needs further investigation.

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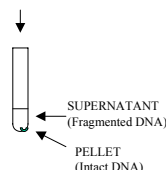
#### ERRATA

**Page 7, SELECTION OF...., text line 4: *start to* should be omitted.**

**Page 15, text line 3 (+ in REFERENCES): *Jansson* should be **Janson**.**

**Page 15, text line 6 from the bottom: (*Natraj and Richards 1993*) should be replaced with (**Curry and Nothnick 1996**).**

**Page 30, Figure 8, (right bottom):** the position of the arrows have been corrected and missing words added.



**Page 44, Figure 21:** The missing words in the right part of the figure can be correctly viewed in Paper IV, Fig. 3.

**Page 49, text line 7: *Andersson et al. 1999* should be included as a reference.**