Apoptosis – the cellular mechanism of rat ovarian follicular atresia
A study on transcriptionally upregulated genes isolated with differential display of mRNA

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Apoptosis – the cellular mechanism of rat ovarian follicular atresia.
A study on transcriptionally upregulated genes isolated with differential display of mRNA

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The normal fate of ovarian follicles is to undergo atresia, and the cellular mechanism behind atresia is apoptosis. This mechanism is often dependent on new mRNA and protein synthesis. To find out if apoptosis in ovarian follicles is dependent on transcription and translation granulosa cells were isolated from preovulatory follicles. The cells were cultured in vitro for 24 h inducing a spontaneous onset of apoptotic DNA fragmentation. Cells incubated in the presence of the transcription inhibitor, actinomycin-D, or the translation inhibitor, cycloheximide, showed a dosedependent decrease in apoptotic DNA fragmentation. This indicates that ovarian cell apoptosis is dependendent on transcription and translation in vitro.

To study genes upregulated during follicular apoptosis, differential display of mRNA was used. Five upregulated genes were isolated in ovaries with a high abundance of apoptosis. Two were unknown and three were homologous to cytochrome b, aldose reductase and β-catenin. In order to characterize these genes, northern blot analysis was performed to ovarian and a prostate model for apoptosis. ARG-33 was expressed in ovary and brain but not in other tissues studied, indicating a possible tissue specific expression. Aldose reductase was upregulated in tissue with a high abundance of apoptosis from three different in vivo models, two ovarian and one prostate model. In addition, aldose reductase was shown using in situ hybridization and immunohistochemistry to be expressed in most follicles of a cycling rat. The functional importance of β-catenin during apoptosis was studied when overexpressed in chinese hamster ovary cells followed by fluorometric detection of fragmented DNA. Interestingly, overexpression of β-catenin induced a 23 % reduction in staurosporin induced apoptotic DNA fragmentation.

In summary, apoptosis in cultured granulosa cells was shown to be dependent on transcription and translation. Differential display of mRNA was shown to be a potent method for the isolation of transcriptionally regulated genes. Several genes were characterized further using northern blot analysis. β-catenin was found to be an inhibitor of apoptosis.

Keywords: atresia, apoptosis, ovary, aldose reductase, β-catenin
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1. INTRODUCTION

The ovary contains follicles, a structure harboring oocytes and producing hormones. These follicles were formed early during embryogenesis to protect and take care of the oocytes, forming the resting pool of follicles. Throughout a human female's life, follicles will leave this pool of resting follicles, to continue to grow and reach ovulation. However, most follicles will not reach ovulation but instead degenerate by a process called atresia. The cellular mechanism for follicular atresia is apoptosis; a form of programmed cell death.

Figure 1. A figure of a mammalian ovary showing the different developmental stages of a follicle, the ovulation and the formation of a corpus luteum. (art by Emil Assarsson)

1.1 Follicular development

During embryogenesis, primordial germ cells migrate from the yolk sac through the dorsal mesentery of the hindgut, to the genital ridge. The germ cells then undergo extensive proliferation, and lose their motile characteristics. In addition, somatic cells, derived from the mesenchyme of the genital ridge, proliferate as well. The somatic cells then organize into cords, which separate and enclose the germ cells to form the pool of resting primordial follicles (Hirshfield 1991).
After the germ cells have ceased mitotic division, meiosis will begin. For the germ cells to become oocytes they have to go through the stages of meiotic prophase, including leptotene, zygotene, pachytene and diplotene. The oocytes then become arrested in the diplotene stage of the first meiotic division where the oocyte will stay until its surrounding follicle leaves the primordial stage and start to grow to reach ovulation. In the pachytene phase, homologous chromosomes recognize each other and line up, so that recombination can occur, and different gene combinations are formed. In the diplotene stage, where the oocyte may halt for several years, multiple transcription occur (Alberts et al. 1994). At this stage, the oocyte may prepare itself for rapid mitosis and implantation, by producing large amounts of mRNA and ribosomes (Picton et al. 1998).

Figure 2. At an early antral stage, the human follicle enters the menstrual cycle. The follicle is now dependent on gonadotropic support for its growth and development. The absence of gonadotropic support results in follicular atresia by an apoptotic cellular mechanism. FSH stimulation results in estrogen production. The increased estrogen production further results in a negative feedback on the hypothalamus and a decreased release of gonadotropins. As the estrogen levels reaches a critical threshold, a positive feedback on the hypothalamus instead results in a peak release of gonadotropins. This gonadotropic peak leads to ovulation. At the time of the gonadotropic peak the follicular cells will differentiate into luteal cells and start to produce progesterone in addition to estrogens. (art by Emil Assarsson)

Follicular growth may begin at any time during the human female's life. A primordial follicle contains an oocyte surrounded by between five and eight flattened somatic cells, the progenitors of follicular granulosa cells. An unknown signal, which may be related to the number of primordial follicles present in the ovary, results in
recruitment of primordial follicles into the early growing phase (Gougeon 1996) (Figure 1). Proliferation of the somatic cells and enlargement of the oocyte characterize initiation of the growing phase. The smallest growing follicles lack an independent blood supply. A single layer of cuboidal granulosa cells surrounds the oocyte in these primary follicles. The follicle continues to grow, resulting in the formation of two theca cell layers, theca interna and theca externa. Small cavities filled with fluid are gathered to form an antrum, resulting in a secondary follicle. A basal lamina will surround the granulosa cells and act as a membrane. There are no blood vessels on the internal side of this membrane. The granulosa cells and the oocyte instead communicate via gap junctions and adherens junctions. The early antral follicles may enter the menstrual cycle, at the size of 2-5 mm in diameter. In the absence of gonadotropins, like for instance before puberty, the early antral follicles instead terminate by atresia. Follicles of this size depend on gonadotropins to support their further development (Gougeon 1996).

Growth is very slow before reaching the stage at which the follicle becomes dependent on gonadotropins. However, when entering the cycle in which they may ovulate, growth continues rapidly (Figure 2). The follicle increases in size from 5 mm to 20 mm in 14 days. Soon after having entered the cycle, the follicles obtain the capability to produce estrogen. The increased expression of estrogen inhibits gonadotropin release, which has been suggested to result in the loss of follicles not yet being able to produce sufficient amounts of estrogen. However, as the estrogen levels increase, an estrogen threshold is reached, which instead of inhibiting gonadotropins induces an immense release of gonadotropins. This peak of gonadotropin release results in ovulation and the release of an oocyte.

1.2 Atresia and Apoptosis

All oocytes present in a woman are formed during embryogenesis. The oocytes multiply by mitosis on their way to the genital ridge, in week 20-24 of pregnancy. At this time, there are about 7 million oocytes present in the ovary. Before birth, a large amount of the oocytes become eliminated, and there are only about 2 million oocytes, contained in follicles, left in the ovary at the time of birth. At the onset of puberty, a large number of the 2 million follicles have been removed by atresia. There are around 400 000 follicles left in the ovary and of these remaining follicles, only about 400 will reach ovulation during the fertile period. At menopause, there are no follicles left in the ovary (Baker 1963). This results in less than 0.1 percentage utilization of the oocytes. The normal physiological fate of the oocytes is therefore to be eliminated by atresia (Table 1).
Morphological characteristics of follicular atresia are for instance, scattered pyknotic nuclei in the granulosa cell layer (Hirshfield 1989), detachment of the granulosa cell layer from the basement membrane (Junquiera et al. 1989), fragmentation of the basal lamina (Bagavandoss et al. 1983), and the presence of cell debris in the antrum of the follicle (Hay et al. 1976). In addition, granulosa cells of an atretic follicle have a reduced synthesis of DNA (Greenwald 1989, Hirshfield 1989) and protein (Byskov 1979). However, the amount of RNA synthesized has been reported to be normal, although some changes are detected in what mRNA is synthesized (Tilly et al. 1992b). In contrast to the granulosa cells, theca cells in the rat, human and rabbit undergo hypertrophy during the first stage of follicular atresia (Braw et al. 1976, Erickson et al. 1985). The oocyte on the other hand undergoes meiosis-like changes (germinal vesicle breakdown), followed by oocyte fragmentation, and disruption of the oocyte-cumulus connection (Tsafriri & Braw 1984). The morphological characteristics of the atretic follicle has been shown to be due to an apoptotic pathway, since DNA of an atretic follicle is fragmented into multiples of 185-200 bp (Zeleznik 1989, Tilly et al. 1991, Wyllie 1980).

### 1.3 Apoptosis

Apoptosis is a general and well-conserved mechanism present in most tissues and species. It is a physiological process, which is necessary for normal embryo development and tissue regeneration. In adult mammals, millions of cells divide into identical daughter cells. It is therefore important that cells could be eliminated in the same rate as mitosis. Apoptosis is a form of programmed cell death, i.e. it involves an intracellular program that differs from the pathological form of death, necrosis (Table 2). Apoptosis is so neat, tidy and rapid that in most tissues only around 1% of cells, found at a specific time, are dead cells. The cell surface of the apoptotic cells

<table>
<thead>
<tr>
<th>HUMAN FEMALE</th>
<th>GERM CELL No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>• gestation week 20</td>
<td>• 7 × 10^6</td>
</tr>
<tr>
<td>• birth</td>
<td>• 2 × 10^6</td>
</tr>
<tr>
<td>• puberty</td>
<td>• 4 × 10^5</td>
</tr>
<tr>
<td>• post-menopause</td>
<td>• 0</td>
</tr>
<tr>
<td>• number ovulated</td>
<td>• 400-500</td>
</tr>
<tr>
<td></td>
<td>&gt; 99.9% are removed</td>
</tr>
</tbody>
</table>
express receptors that can be recognized by surrounding cells, which rapidly
phagocytose and digest apoptotic bodies formed by the dying cell. The phagocytosis
occurs without leakage of cellular constituents, without inflammation, and without
damage to surrounding cells (Anderson et al. 1997).

Table 2.

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affects scattered individual cells</td>
<td>Affects tracts of continuous cells</td>
</tr>
<tr>
<td>Chromatin &amp; cytoplasmic condensation, cell shrinkage</td>
<td>Cell swelling and rupture of plasma membrane</td>
</tr>
<tr>
<td>Normal ATP level</td>
<td>Decreased ATP level</td>
</tr>
<tr>
<td>No inflammation</td>
<td>Elicits inflammatory responses</td>
</tr>
<tr>
<td>Apoptosis specific DNases</td>
<td>Activation of nonspecific DNases</td>
</tr>
<tr>
<td>May require mRNA and protein synthesis</td>
<td>Not dependent upon new mRNA or protein synthesis</td>
</tr>
</tbody>
</table>

1.3.1 Historical background

Although the essence of programmed cell death in the morphology of embryo
development has been known for decades (Glücksmann 1951), it has not been
accepted as a common cell-fate until in 1972 (Kerr et al. 1972). Now, however,
apoptosis has been shown to be involved in general cell deletion. In for instance
diseases like cancer, heart disease, stroke, AIDS, autoimmunity, degenerative
diseases, the symptoms could often be ascribed to pathological increase or decrease
in activity of apoptosis (Miller & Marx 1998, Barinaga 1998a, b). It is therefore of
major interest to learn more about apoptosis, and perhaps in the future be able to
manipulate the process.

Programmed cell death has been called many things over the years. In 1972, Kerr
talked to Professor James Cormack of the department of Greek, University of
Aberdeen, about a name for their findings on “shrinkage necrosis” (Kerr 1965, Kerr
1971). Other groups have reported the process under a variety of names:
Councilman bodies in the liver (Klion & Schaffner 1966); tingible bodies in the
germinal centers of lymph nodes (Swartzendrubber & Congdon 1963); dyskeratotic
cells in the skin after ultraviolet radiation damage (Wilgram et al. 1970) and karyolytic
bodies in the epithelium of duodenal crypts after whole body irradiation of mice
(Hugon & Borgers 1966). Professor Cormack suggested the word “apoptosis”,

Table 2.
which is used in Greek to describe the “dropping off” or “falling off” of petals from flowers or leaves from trees.

1.3.2 The cellular mechanism of apoptosis

Apoptosis has been intensively studied in *C. elegans*. Indeed, many epoch-making results have been found in *C. elegans*. Three of the genes shown to be essential for developmental programmed cell death in *C. elegans* are ced-3, ced-4, and ced-9 (Ellis & Horvitz 1986, Yuan & Horvitz 1990, Hengartner et al. 1992). Inactivation of ced-3 or ced-4 results in survival of cells otherwise destined to be deleted during development. Ced-9, on the other hand, is a survival factor, which inhibits apoptosis in cells that are not destined to die. A large number of other genes involved in apoptosis have been isolated in *C. elegans*. The genes seem to be involved in different stages of apoptosis and a schedule has been drawn out in an elegant manner to clarify the pathways of apoptosis (Ellis et al. 1991). These first studies in *C. elegans* have contributed to the finding of mammalian homologues. For example, the mammalian homologues of ced-9, ced-4 and ced-3 have been shown to be members of the Bcl-2 gene family, Apaf-1 and members of the caspase gene family, respectively. The isolation of these molecules has resulted in extensive progress in finding the intracellular mechanism of apoptosis.

The initiation of apoptosis is tissue and species specific, and not all cells will be eliminated in response to the same stimulus. Apoptosis in thymocytes is activated by corticosteroids, while the same substance inhibits apoptosis in other cells (Kiess & Gallaher 1998). Irradiation or drugs often result in the triggering of a p53 dependent pathway, initiating apoptosis. Some cells express Fas or other death receptors on their surface, initiating a complex death signal via DISC-formation.

Caspases play a central role in the initiation, final decision and execution of apoptosis (Table 3). The name caspase originates from its two catalytic activities. The “c” stands for its cysteine protease activity and the “aspase” reflects its ability to cleave after an aspartic acid (Alnemri et al. 1996). Several caspases have been isolated but although they all cleave after an aspastic acid, each enzyme’s activity is specific. Caspases are synthesized as inactive proenzymes composed of three domains. They include an amino-terminal domain, a small subunit (10 kDa) and a large subunit (about 20 kDa). The enzyme is activated by proteolytic cleavage at two sites,
between the prodomain and the large subunit and between the large and the small subunits. Caspases can be divided into two groups, caspases with a long prodomain (caspases-1, -2, -4, -5, -8, -9, -10, -11, -12, and -13) and caspases with a short prodomain (caspases-3, -6, -7, -14) (Table 3). Caspases with a long prodomain usually act more proximally in the initiation of cell death by for example activating downstream caspases. They are therefore called initiator or upstream caspases. Caspases with a short prodomain act more distally to the initiation of apoptosis by cleaving specific death substrates (e.g. poly(ADP) ribose polymerase (PARP), inhibitor of caspase-activated DNase (ICAD), gelsolin and lamins). They are therefore called effector or downstream caspases. The prodomain of several initiator caspases (procaspases-1, -2, -4, -5, -9, -11, -12, and -13) contain a CARD domain (caspase recruitment domain). The prodomain of other caspases (caspases-8 and -10) instead contain a DED domain (death enhancer domain). These domains have similar three dimensional structures and can bind other molecules containing these structures. In fact, the adhesion of prodomains of initiator caspases to each other is of major importance for caspase activation. Binding of a ligand to a death receptor first initiates adhesion of molecules containing a death domain (DD) and then the adhesion of long prodomains containing DED and CARD domains, resulting in the formation of the DISC (death-inducing signal complex) and the activation of downstream caspases, e.g. caspase-3. Another caspase, caspase-9, is activated by the recruitment into a complex of Apaf-1 and cytosolic cytochrome c. Activation of initiator caspases therefore primarily seem to occur by recruitment and complex formation (Li & Yuan 1999).

Table 3. List of caspases, their activity (initiator or effector), synonym names and original references.

<table>
<thead>
<tr>
<th>Caspase</th>
<th>Activity</th>
<th>Synonyms</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>caspase-1</td>
<td>CARD</td>
<td>ICE</td>
<td>Cerretti et al. 1992, Thornberry et al. 1992</td>
</tr>
<tr>
<td>caspase-2</td>
<td>CARD</td>
<td>ICH-1, Ned2</td>
<td>Kumar et al. 1993, Wang et al. 1994</td>
</tr>
<tr>
<td>caspase-4</td>
<td>CARD</td>
<td>TX, ICH-2, ICE&lt;sub&gt;rel&lt;/sub&gt;II</td>
<td>Munday et al. 1995, Kamens et al. 1995, Faucheu et al. 1995</td>
</tr>
<tr>
<td>caspase-5</td>
<td>CARD</td>
<td>ICE&lt;sub&gt;rel&lt;/sub&gt;III, TY</td>
<td>Munday et al. 1995</td>
</tr>
<tr>
<td>caspase-6</td>
<td>Downstream</td>
<td>Mch2</td>
<td>Fernandez-Alnemri et al. 1995a</td>
</tr>
<tr>
<td>caspase-9</td>
<td>CARD</td>
<td>ICE-LAP6, Mch6</td>
<td>Srinivasula et al. 1996, Duan et</td>
</tr>
</tbody>
</table>
The Bcl-2 family members have been shown to play a central role in determining whether the cell should be eliminated by apoptosis or not. The members of the Bcl-2 family can be divided into two groups, the death antagonists (Bcl-2, Bcl-X\textsubscript{L}, Bcl-w, Bfl-1, Brag-1, Mcl-1, A1) and the death agonists or proapoptotic proteins (Bax, Bak, Bcl-X\textsubscript{S}, Bad, Bik, Bid, Hrk, Boo and Egl-1 (C.Elegans)). Most of this family’s members contain a transmembrane domain (exceptions are e.g. Bad, Bid and Egl-1) and are situated in the outer mitochondrial membrane. The proapoptotic proteins contain a Bcl-2 homology (BH) domain 3, which seem to be essential for their proapoptotic activity. In addition, some of the proapoptotic proteins also contain 2 or 3 additional BH domains (Bax, Bad and Bak). Proapoptotic Bcl-2 family members seem to be activated in multiple ways. Egl-1, Hrk and Bax have been shown to be transcriptionally upregulated. Bad has been shown to be phosphorylated before it is translocated from the cytoplasm to the mitochondrial membrane. Bid is cleaved by caspase-8 after which it is translocated to the mitochondria. The movement of proapoptotic proteins, containing a BH3 domain from the cytoplasm to the mitochondria, has been shown to occur during apoptosis. The Bcl-2 family inhibitors of apoptosis may act by heterodimerization with proapoptotic proteins in the mitochondrial membrane (Li & Yuan 1999). The antagonists and agonists have been shown to be able to either heterodimerize or homodimerize with each other, and the net result of this dimerization can result in either apoptosis or the inhibition of the apoptotic stimulus (Kroemer 1997). For instance, Bcl-2 and Bcl-X\textsubscript{L} heterodimerize with Bax. Homodimerized Bax has been shown to form a pore in the mitochondrial membrane. Bcl-2, and Bcl-X\textsubscript{L} are able to block this pore formation by inhibiting the homodimerization of Bax (Yang et al. 1997, Skulachev et al. 1998). The pore opens a channel for the release of different mitochondrial proteins, e.g. cytochrome c and apoptosis inducing factor (AIF). The release of these factors has also been suggested to occur as a result of permeability transition pore opening, causing rupture of mitochondrial membrane potential ($\Delta\Psi_m$).
Cytochrome c is a small, very stable hemeprotein, containing a covalently bound heme c as a prosthetic group. It binds to the outer surface of the inner membrane of mitochondria and is known to transport electrons from cytochrome c₁ to cytochrome oxidase during respiration. When cytochrome c is released out in the cytoplasm, it participates in the induction of apoptosis by activating procaspase-9. To do this it cooperates with apoptosis-activating factor 1 (Apaf-1). This is a cytoplasmic protein that possesses a nucleotide binding site for dATP, a cofactor of apoptosis. Both procaspase-9 and Apaf-1 contain CARD domains, by which they can attract each other, resulting in the proteolytic cleavage of procaspase-9. This activation complex containing Apaf-1, caspase-9 and cytochrome c is commonly known as an “apoptosome”. Caspase-9 then activates pro-caspase-3 (Kluck et al. 1997, Motyl 1999) (Figure 3).

Figure 3. Oversimplified figure summarizing some of the today known events of apoptosis. Apoptosis may be initiated by a variety of signals. Shown are the physiological pathways via the death receptors inducing the formation of a DISC (death-inducing signal complex) or via hormonal activity resulting in transcriptional regulation. Other non-physiological stimuli are for instance irradiation resulting in upregulation of p53, or anticancer drugs e.g. staurosporin. After initiation, apoptosis seems to involve more common events like for instance mitochondria, nucleus and phagocytosis. The decision to go through with the apoptotic death program involves the mitochondria and the Bcl-2 family members. Proapoptotic members of the Bcl-2 family become activated and translocated to the mitochondrial membrane. In the mitochondrial membrane the proapoptotic members induce pore opening and the release of e.g. cytochrome c (cytochrome c) and apoptosis inducing factor (AIF) resulting in the formation of an apoptosome. The apoptosome is composed of Apaf-1 and cytochrome c and will retrieve procaspase-9, which, when recruited, is activated. Anti-apoptotic members of the Bcl-2 family can inhibit the formation of an apoptosome, possibly by heterodimerizing with proapoptotic molecules in the mitochondrial membrane. Caspase-9 has been shown to induce proteolytic cleavage of inhibitor of caspase-activated DNase (ICAD), resulting in the release of CAD, cleaving the DNA between the nucleosomes into multiples of 185-200 bp - the well known apoptotic laddering pattern. In addition to nuclear events, other events are activated, resulting in the final phagocytosis of cellular contents by nearby lying cells or macrophages.
The activation of caspase-3 has been shown to induce nuclear degradation. DNA degradation occurs in three steps. The first step includes degradation of DNA into fragments of 300 kbp. The next step degrades DNA into fragments of 50 kbp, and the final step degrades DNA into the well known multiples of 183-200 bp, which has long been a hallmark of apoptosis (Walker et al. 1993). A nuclease capable of internucleosomal cleavage of DNA was recently cloned. Caspase-activated DNase (CAD) is localized to the cytoplasm in an inactive form bound to ICAD (inhibitor of CAD). ICAD possesses a caspase-3 cleavage site. Caspase-3 is therefore capable of activating DNA fragmentation by proteolytic cleavage of ICAD and thereby releasing CAD from ICAD (Enari et al. 1998, Sakahari et al. 1998). A human homologue of mouse CAD has been found, called DNA fragmentation factor 45 (DFF45) (Liu et al. 1997) (Figure 3). Other nuclear events include DNA margination, chromatin condensation, proteolytic cleavage of lamin and PARP (poly(ADP-ribose)polymerase).

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 by nearby lying cells or macrophages. The release of the apoptotic cell from surrounding cells has been shown to be caused by disassembly of focal adhesion complexes. At the same time, actin rearranges to the cellular membrane, resulting in rounding up of the cell. Focal adhesion proteins, including p130CAS and focal adhesion kinase (FAK) have been shown to be cleaved by caspases. Other proteins involved in linking actin with the plasma membrane (α-actinin, fodrin and talin) are cleaved by calpains. This indicates the importance of caspases and calpains, also in the final events of apoptosis. After the cell is rounded up, it starts to bleb. This process seems to involve myosin-II, which contracts the peripheral ring of actin. A concomitant weakening of the membrane-actin linkages in some areas results in blebbing. This is a highly energy demanding activity which
eventually stops. The cessation of blebbing results in condensation of the cell and formation of apoptotic bodies (Mills et al. 1999).

The recognition of a dead apoptotic cell by neighboring cells and non-activated macrophages has been intensively studied over the last few years. Apoptotic cells seem to show up a “flag” to tell other cells “come and eat me”, and one of these flags are phosphatidylserine. Phosphatidylserine is normally present on the cytoplasmic side of the cell membrane. However, when the cell undergo apoptosis, phosphatidylserine flips over to the external face of the bilayer. Macrophages and nearby lying cells are able to recognize the lipid head by a receptor, triggering phagocytosis (Shiratsuchi et al. 1997). Recently, scavenger receptor class B type I (SRBI) expressed in sertoli cells and theca cells was shown to recognize apoptotic spermatogenic cells and granulosa cells, respectively (Shiratsuchi et al. 1999, Svensson et al. 1999). Previous results have shown that SRBI recognize externalized phosphatidylserine (Fukasawa et al. 1996) (Figure 3).
1.4 Apoptosis in the ovary

In the ovary, apoptosis is the cellular mechanism removing all but few growing follicles during their way to ovulation. Apoptosis is also the mechanism behind removal of corpus luteum during luteolysis (Rueda et al. 1995, Roughton et al. 1999, Bowen et al. 1999).

The initiation of apoptosis in granulosa cells of antral follicles seem to be controlled by hormones, acting via paracrine and/or autocrine intraovarian signals. Previous results have shown that a decrease in the levels of circulating gonadotropins, either by hypophysectomy or a blockade of the LH/FSH surge, leads to massive ovarian atresia (Ingram, 1953; Braw & Tsafriri, 1980). Apoptosis in the ovary induced by hypophysectomy can be reduced with FSH treatment in vivo. In addition to FSH, estrogen also inhibits hypophysectomy induced apoptosis in the ovary, while androgens instead antagonize the effect of estrogen (Billig et al. 1994, Ingram 1959, Billig et al. 1993).

The intraovarian signaling system has been studied in vitro demonstrating several growth factors to take part in this mechanism. Growth factors are important factors involved in ovarian development and function (Ojeda & Dissen 1994). The IGF-IGFBP system has been shown to take part in the regulation of apoptosis. This system also take part in cell proliferation, aromatase activity and progesterone biosynthesis (Yoshimura 1998). IGF-I inhibits granulosa cell apoptosis in early antral and preovulatory follicles (Chun et al. 1994, Chun et al. 1996). However, IGF-I is not capable of inhibiting spontaneously induced apoptosis in preantral follicles (McGee et al. 1997). This effect of IGF-I, in early antral and preovulatory follicles, is counteracted by IGFBP-3. IGFBPs are known to modulate biologically active IGF-I in the ovary (Adashi et al. 1985). Gonadotropins may act to inhibit apoptosis in the granulosa cells by reducing the expression of IGFBPs and increase the expression of IGF-1 (Liu et al. 1993, Hernandez et al. 1989). The direct effects of growth hormone on the ovary are mediated via local IGF-I production (Davoren & Hsueh 1986). GH has been shown to suppress apoptosis in preovulatory follicles but not in early antral follicles (Eisenhauer et al. 1995). This action was counteracted in the presence of IGFBPs. EGF and bFGF also inhibit granulosa cell apoptosis of preovulatory follicles (Tilly et al. 1992a). EGF and bFGF have been shown to be upregulated by gonadotropins, but the presence of a receptor is essential for their action. The receptors are primarily present at the time of ovulation, which can explain why EGF and bFGF only have a minimal effect on suppression of apoptosis in early antral follicles (McGee et al. 1997). Activin inhibits granulosa cell apoptosis in a dose-dependent manner and the expression of activin subunits increase with the size of the follicle. The maximal suppression effect is reached at the size of an early antral
follicle (Mayo 1994, Chun et al. 1996). Follistatin inhibits the action of activin (Findlay 1993). In addition to growth hormones, the cytokine IL-1β has been shown to inhibit apoptosis (Chun et al. 1995). LH may stimulate the production of IL-1β which results in an increased synthesis of nitric oxide (NO). NO acts via the second messenger, cGMP to suppress apoptosis (Chun & Hsueh 1998).

Inducers of apoptosis in the granulosa cells are e.g. GnRH, androgens, IL-6, ROS and TNF-α (Billig et al. 1994, Billig et al. 1993, Gorospe & Spangelo 1993, Wong et al. 1989, Tilly & Tilly 1995, Kaipia et al. 1996). TNF-α inhibits the survival effect of FSH and uses ceramide as a second messenger in the sphingomyelin signaling pathway to induce apoptosis (Jarvis et al. 1993). The Fas/Fas-L death receptor system is present also in granulosa cells and may involve intracellular adhesion, forming a DISC. However, this has not yet been studied in the ovary. The Fas/Fas-L system contributes to immune privilege displayed by testis and Sertoli cells and may also be part of immune privilege in the ovary (Streilein 1995).

Different autocrine and paracrine intraovarian pathways, activated by gonadotropins, may in this way inhibit apoptosis. Other systems instead inhibit the survival factors to induce apoptosis. However, the concluding signal results in intracellular actions similar to more general mechanisms involved in apoptosis. The proapoptotic Bcl-2 family members, e.g. Bax and Bad, trigger pore opening in the mitochondria resulting in the release of e.g. cytochrome c. This results in the activation of caspases, DNA degradation and nuclear condensation (Robles et al. 1999). Interestingly, many of the members of the Bcl-2 family have been isolated in the ovary. Indeed, a few of them are expressed primarily in the ovary. The anti-apoptotic Boo (Bcl-2 homologue of ovary) expression is highly restricted to the ovary and the epididymis and Bok (Bcl-2 related ovarian killer) is highly expressed in the ovary, testis and uterus (Song et al. 1999, Hsu et al. 1997a). Other members of the Bcl-2 family, which were isolated in the ovary are Bad (Bcl-XL/Bcl-2 associated death promoter) and Bod (Bcl-2 related ovarian death gene) (Hsu et al. 1997b, Hsu et al. 1998). Bod is however more widely expressed in multiple tissues.

Although the major site of apoptotic DNA fragmentation is in the granulosa cell layer, scattered apoptotic theca cells can be detected in situ (Palumbo & Yeh 1994). During proliferation, theca cells provide granulosa cells with molecules necessary for their proliferation and function, through the basal lamina. Theca cells may take part in the phagocytosis of granulosa cells, because theca cells express SRBI, that recognize PS cell surface exposure in apoptotic granulosa cells, making attachment possible (Svensson et al. 1999). Apoptosis seem to be delayed in theca cells (Logothetopulos 1995), but eventually will eliminate also this celltype from the ovary (Palumbo & Yeh 1994). The mechanism behind apoptosis in theca cells is
currently under investigation, but Bcl-2 and caspase-1 have been shown to be involved (Foghi et al. 1998). This indicates that theca cell apoptosis involves the same intracellular signaling system as most cells, i.e. the Bcl-2 family and the caspases. However, the induction of apoptosis in theca cells differ from granulosa cell apoptosis, since TGF-α and TGF-β in combination have been shown to induce apoptosis in cultured theca cells (Foghi et al. 1997).

Granulosa cells are the first to be affected by apoptosis during the hormonally regulated phase of folliculogenesis. However, at the primordial and preantral stage of follicular development, the first cell to be affected is the oocyte (Driancourt & Thuel 1998, Morita et al. 1999). The mechanism involved in the induction of oocyte apoptosis seems to involve paracrine factors. Oocytes, not incorporated into primordial follicles, are eliminated at 3-4 days of age (Ohno & Smith 1964). This elimination may depend on the inability of these oocytes to control growth and maturation. Key molecules in this process are e.g. EGF/TGF-α, FGF, inhibin/activin and c-kit/KL (Driancourt & Thuel 1998). The death machinery of apoptosis in the oocyte has been shown to involve the Bcl-2 family, since Bax deficient mice show a delayed menopause and mice lacking functional Bcl-2 show a decrease in the amount of primordial follicles (Perez et al. 1999, Ratts et al. 1995). In addition, oocytes from Bax deficient mice show resistance to apoptosis induced by chemotherapy (Perez et al. 1997).
2. AIMS OF THE STUDY

The expression of proteins in a cell, regulates its' fate and destination. Gene expression can be regulated in multiple ways, e.g. control of transcription rate, processing of RNA, RNA transport, degradation of RNA, translation and protein activity. It is therefore interesting to note that in several tissues, genes are transcriptionally upregulated during apoptosis (Amson et al. 1996, Osborne 1995, Owens & Cohen 1992, Zhang & Zhang 1996). In fact, transcription and translation has in many celltypes been shown to be necessary for apoptosis to occur. On the other hand, transcriptional- and translational inhibitors has also been shown to induce apoptosis in some cells.

In the ovary, several genes have been shown to be upregulated during apoptosis. For instance, IGFBPs are located to atretic follicles (Nakatani et al. 1991; Erickson et al. 1992). Testosterone repressed prostate message-2 (TRPM-2) (Kaynard et al. 1992), angiotensin-II (Daud et al. 1988), and cathepsin-D (Dhanasekaran & Moudgal 1989) are other genes that are upregulated during follicular apoptosis. In contrast, aromatase, and gonadotropin receptors are downregulated during apoptosis in the follicles (Tilly et al. 1992b). The total amount of mRNA expression detected in apoptotic compared to normal follicles does not change, since ribosomal RNA, and β-actin mRNA levels are constant (Tilly, et al., 1992). These data suggested that apoptosis in ovarian follicles is regulated by transcription and translation.

The aims of the present study were:

• to determine if ovarian follicular apoptosis is regulated by transcription and translation.

• to evaluate the gene expression array, differential display of mRNA.

• to isolate and characterize genes transcriptionally upregulated during apoptosis in the ovary.
3. Methodology

This section is a brief description of the main methods used in this thesis. The methods are described in more detail in the individual papers.

3.1 Animals

Adult cycling, immature female, mature male (B-K, Stockholm, Sweden), and hypophysectomized immature (Möllegård, Denmark) rats were kept under controlled environmental conditions with free access to tap water and pellets. The rats were kept under controlled environmental conditions, with lights on between 0500-1900 h and with 55-60 % relative humidity. All experiments were approved by the local animal ethical committee.

3.1.1 DES-in vivo ovarian apoptosis model

Hypophysectomized immature rats were treated with the estrogen analogue, diethylstilbestrol. To achieve a low abundance of apoptosis in the ovaries, the rats were given an additional treatment with FSH on day three and four after hypophysectomy (+DES). High abundance of apoptosis was achieved by removing the estrogen implant on day two after hypophysectomy, leaving the animal without treatment for an additional two days (-DES) (Billig et al. 1993, Billig et al. 1994, Chun et al. 1996).

3.1.2 PMS-in vivo ovarian apoptosis model

Immature rats (25-26 days old) were given a 10 IU PMS treatment. This treatment induces a superovulation, controlled by light/dark conditions and the maturity of the animal. A large amount of follicles are saved from atresia at an early antral stage and can reach ovulation, using this technique. This results in reduced apoptosis noticed in PMS stimulated animals (+PMS) compared to immature non-stimulated animals (-PMS) (Bicsak et al. 1986, Tilly et al. 1992a).

3.1.3 In vitro granulosa cell apoptosis

Granulosa cells were isolated from preovulatory follicles, isolated from PMS stimulated (10 IU) immature rat ovaries (27-28 days old), 48 h after treatment. The
incubation of the cells in medium results in a spontaneous onset of apoptosis (Tilly et al. 1992a).

### 3.1.4 In vivo prostate apoptosis model

Male adult rats were castrated, and the ventral prostate was isolated on day 0, 1, 2, and 3 after castration. (Kyprianou & Isaacs 1988).

Figure 4. Flowchart showing the major steps of differential display of mRNA. RNA is isolated from two (or more) different groups of tissue or cells. The tissue/cells are carefully chosen to select for the physiological condition, which is being studied on a transcriptional level. Reverse transcription (RT) is performed on the RNA using an anchored poly-T primer (T\textsubscript{12}MN). The same anchored poly-T primer in addition to an arbitrary decaprimer is used in the subsequent polymerase chain reaction (PCR). The PCR is run at low temperature in the presence of radioactively labeled dATP. The product is then visualized on a denaturing polyacrylamide gel, showing a cDNA footprint. The different groups of cDNA show an almost matching footprint. However, some differences can be found. These differences are chosen. Further work includes excising the different cDNA clone, reamplification, subcloning and use as probe to back-hybridize to RNA from tissue treated as the tissue used in the differential display assay.
3.2 Differential display of mRNA

Differential display of mRNA was performed as described previously (Liang & Pardee 1992) (Figure 4). Total RNA isolated as described previously (Chomczynski & Sacchi 1987) was reverse transcribed using an oligo (dT)-anchored primer. The resulting cDNA was further amplified using PCR in the presence of corresponding oligo (dT)-anchored primer, an arbitrary primer and \([^{35}\text{S}]\text{-dATP}\. Subsequently, the cDNA was separated by electrophoresis on a denaturing polyacrylamide gel, and visualized by autoradiography. Differentially expressed cDNAs were excised from the gel, eluted in boiling water for 15 min, precipitated, and reamplified, using the same primers as were used in the differential display of mRNA reaction. The cDNA fragments were subcloned into the PCR II vector (Invitrogen) and sequenced using a cycle-sequencing kit (Amersham). Subcloned cDNAs were further used as probes for northern blot analysis.

3.3 Northern blot hybridization assay

10 µg RNA was separated by electrophoresis on a 1 % agarose gel containing 6 % formaldehyde and MOPS buffer (Sambrook et al. 1989). The RNA was then transferred to a Hybond-N nylon membrane (Amersham) in the presence of 20 × SSC using a vacuum-blotting unit. The nucleic acid was cross-linked to the nylon membranes by baking the membranes at 80°C for 3h. Membranes were prehybridized for two hours followed by hybridization with a randomly primed cDNA probe over night. The following day, membranes were washed. Temperatures and washing procedure was optimized for the different cDNA clones isolated with differential display of mRNA. The rinsed filters were exposed to Hyperfilm MP films (Amersham). Autoradiograms were digitized using an Arcus II (AGFA) scanner, and Adobe Photoshop 3.0 software.

3.4 Cell Culture and Transfection of CHO cells with β-catenin

Chinese hamster ovary (CHO) cells were plated (2×10^5 cells/plate), and allowed to adhere overnight. After attachment, the medium was changed, and the cells were transfected, using the lipofectamine plus procedure (Gibco), with mouse β-catenin, Bcl-2, both subcloned into the pcDNA3 vector, or with the pcDNA3 vector alone (Invitrogen, Groningen, the Netherlands). To induce apoptosis in the CHO cells, 0.2 µM staurosporin (Sigma-Aldrich), diluted in DMSO, was added to the culture plates 24 h after transfection with plasmids. The cells were harvested 0, 3 and 5 h
after addition of staurosporin, after which the cells were collected by centrifugation. To determine the amount of apoptotic cells, DNA was isolated. Fragmented apoptotic low molecular weight DNA isolated from the DNA preparation after removal of intact non apoptotic nuclei was measured with fluorescence spectrophotometer (Wyllie 1980, Billig et al. 1998, Labarca & Paigen 1980). In addition, low molecular weight DNA was labeled with $^{35}$S-dATP (Amersham), using terminal transferase (Boehringer Mannheim), and was fractionated through 1.8 % agarose gels, as described previously (Billig et al. 1998), identifying the characteristic apoptotic DNA ladder. The experiment was repeated three times with two culture plates per time and treatment. Results are presented as mean±SEM (n=6), and Anova followed by Student-Newman-Keuls multiple range test were used for statistical analysis.
4. RESULTS AND COMMENTS

4.1 The role of mRNA and protein synthesis in granulosa cell apoptosis

The physiology of a cell is regulated by the expression and concentration of its protein content. Proteins may be up- or downregulated by transcriptional and/or posttranscriptional mechanisms. Apoptosis, in many cells and tissues, has been shown to be influenced by transcription and translation (Martin 1993). The increased and decreased expression of a number of genes during apoptosis in the ovary, support the hypothesis that also apoptosis in the ovary is regulated by alterations in the transcription rate (Hsueh et al. 1994). To confirm the influence of transcription and translation on apoptosis in the ovary, granulosa cells were incubated in the presence of the transcription inhibitor, actinomycin-D and the translation inhibitor, cycloheximide. Granulosa cells spontaneously undergo apoptosis when incubated in vitro. However, in the presence of either actinomycin-D or cycloheximide, the spontaneous onset of apoptosis was dose-dependently inhibited (Paper I). Indeed, this indicates that new protein synthesis is of major importance for granulosa cell apoptosis in vitro and may be important also for ovarian apoptosis in vivo.

4.2 Isolation of transcriptionally upregulated genes in rat ovarian tissue with a high abundance of apoptosis

Having supported the theory on transcriptional importance for apoptosis in the ovary, the next goal was to isolate genes involved in this process. To do this, we used differential display of mRNA (Liang & Pardee 1992). The mRNA expression was compared in two pools of ovarian RNA. One pool contained ovarian RNA with a low abundance of apoptosis, isolated from hypophysectomized rats treated with estrogen and FSH (+DES). In comparison, the other pool contained ovarian RNA with a high abundance of apoptosis, isolated from estrogen substituted, hypophysetomized rats, two days after estrogen withdrawal (-DES). The combination of 4 poly-T primers and 20 decaprimer resulted in the isolation of 181 cDNA clones with differential expression (Paper I) (Table 4). The genes were either upregulated in ovaries with a high abundance of apoptosis (72 cDNA clones), or in ovaries with a low abundance of apoptosis (109 cDNA clones). The latter 109 cDNA clones are still waiting to be studied. However, the remaining 72 cDNA clones found in ovaries with a high abundance of apoptosis, were subcloned and sequenced, indicating a variety of interesting genes, some unknown and some never.
previously studied in the ovary. Three cDNA clones turned out to be identical, leaving 69 individual cDNA clones.

Although these data were interesting indeed, the upregulation of these genes, found in the differential display assay, needed to be confirmed. The confirmations were performed using northern analysis on RNA from the same ovarian model for apoptosis as was used in the differential display assay. However, a large amount of the cDNA clones found, were not visible on the northern blot, indicating a very low transcription rate. In fact, of the 69 cDNA clones, only 27 cDNA clones were above detection limit when using northern blot analysis. In addition, some genes upregulated in the differential display assay, were “false positive clones”, and no difference in transcription rate was detected on northern blots. However, five cDNA clones were differentially expressed also when using northern blot analysis. These genes were designated ARGs for apoptosis related genes.

Table 4. cDNA clones isolated using differential display of mRNA

<table>
<thead>
<tr>
<th>Ovary</th>
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<tbody>
<tr>
<td>Totally 181 cDNA</td>
</tr>
<tr>
<td>Apoptotic 69 cDNA 3 duplicates</td>
</tr>
<tr>
<td>5 -higher expression in apoptotic</td>
</tr>
<tr>
<td>2 unknown</td>
</tr>
<tr>
<td>3 known: -cytochrome b -aldose reductase -beta-catenin</td>
</tr>
<tr>
<td>27 -no difference in expression or false positives</td>
</tr>
<tr>
<td>37 -below detection</td>
</tr>
<tr>
<td>Non-apoptotic 109 cDNA</td>
</tr>
</tbody>
</table>

4.3 Sequence homologies and tissue distribution

The genes found to be differentially expressed not only using differential display of mRNA, but also using the northern blot hybridization assay, were in most cases
identified when compared to known genes in the NCBI gene databank. However, in some cases the gene did not match any known genes in the NCBI databank. ARG-33 was one of the genes not identified, and possibly a novel protein, expressed only in ovary and brain of the tissues studied. ARG-33 was not found in testis, muscle, heart, placenta, lung, kidney and adrenal glands (Paper I). Recently, several EST homologues of ARG-33 were found in the gene databank (accession A1713775; A1763758; A1711454; A1230470; A1577353; AA997026). These EST clones, isolated from a Rattus norvegicus normalized atrium, embryo, or whole-eye library, showed high homology to ARG-33. The homology was 99-100% over most of the sequence. However, ARG-33 was missing an internal base sequence of about 30 bp (bp 152-183). The function of those EST clones are currently unknown.

ARG-9 showed high homology to the ankyrin binding cell adhesion molecule, neurofascin. In contrast to other genes isolated using differential display of mRNA, ARG-9 was homologous to the 5'-untranslated end of neurofascin. In addition, the tissue expression pattern indicated that the genes were not identical. Neurofascin was found only in the brain and in the spinal cord, while ARG-9 was found in all tissues studied. Most expression was found in the brain, ovary, uterus, muscle and lung (Paper II).

The remaining three cDNA clones were known according to the NCBI gene databank. ARG-40 was homologous to mitochondrial cytochrome b, and was expressed in all tissues studied (Koike et al. 1982). Highest expression level was detected in heart and brain (Paper I). ARG-47 was homologous to aldose reductase, and was expressed in all tissues studied but liver. Highest expression level was detected in ovary, uterus, testis, adrenals and heart (Paper III). Finally, ARG-8 revealed β-catenin to be upregulated in ovaries with a high abundance of apoptosis. β-catenin was also expressed in all tissues studied, although highest level of expression was detected in ovary, adrenals and brain (Paper IV).

### 4.4 Transcriptional regulation in additional ovarian models and a prostate model for apoptosis

The isolation of genes that were found to be transcriptionally upregulated in ovaries with a high abundance of apoptosis, induced by withdrawal of the estrogen support of hypophysectomized rats, was further studied. However, this upregulation could be a result of the treatment as well as the induction of apoptosis. To further support the relationship of the upregulated genes and apoptosis in the ovary, the gene expression was studied in additional ovarian and a prostate model for apoptosis.
In one model, the high abundance of apoptosis, detected in prepubertal rat ovaries, was inhibited by treatment of the rats with PMS (pregnant mare’s serum gonadotropin). Interestingly, only aldose reductase, of the genes studied, showed higher expression in immature rat ovaries (i.e. high level of apoptosis) compared to ovaries from PMS treated rats (i.e. low level of apoptosis) (Paper III).

In addition to the in vivo models for apoptosis in the rat ovary, a model for apoptosis in vitro was used, i.e. cultured rat granulosa cells undergo spontaneous onset of apoptosis in vitro. This is the same model as was used when studying the effect of transcription- and translation inhibitors on apoptosis. The idea behind this model was to discriminate for upregulation in the granulosa cells or in the residual ovary. However, none of the genes found to be upregulated in the DES-ovarian apoptosis model, were upregulated after induction of apoptosis in cultured granulosa cells. In fact, ARG-33 was below detection limit, which may indicate that it is not expressed in granulosa cells, but in the residual ovary (Papers I, II and IV).

In addition to models for apoptosis in the ovary, the cDNA clones were studied in the prostate after induction of apoptosis by castration. Interestingly, aldose reductase, and ARG-40 was upregulated on day two and three, respectively, after induction of apoptosis. ARG-33 was below detection limit (Paper I and III).

4.5 Morphological studies on aldose reductase

The aldo-keto reductase family member, aldose reductase, was studied further. In situ hybridization studies on ovaries from cycling rats, revealed high expression of aldose reductase in granulosa cells of all stages of development. High expression of aldose reductase was present in follicles with apoptotic DNA fragmentation, as well as in healthy follicles. However, a few follicles did not show any expression (Paper III).

4.6 Functional studies on β-catenin

The role of β-catenin in apoptosis was studied in CHO cells. β-catenin was overexpressed, and the effect on apoptosis was determined by measuring the amount of low molecular weight DNA fluorometrically and radiolabelled as fragmented DNA on an agarose gel. However, β-catenin did not affect the levels of fragmented DNA, when overexpressed. To induce apoptosis in CHO cells, the cells were incubated in the presence of staurosporin. Interestingly, concomitant overexpression of β-catenin resulted in 23 % less DNA fragmentation compared to mock transfected cells in the presence of staurosporin. As a control, CHO cells overexpressing the inhibitor of apoptosis, Bcl-2, displayed a 40 % reduction in staurosporin induced DNA fragmentation. Indeed, this indicates that β-catenin is
important for the inhibition of apoptosis in staurosporin treated CHO cells (Paper IV).
5. DISCUSSION

5.1 Methodological considerations

The human genome contains approximately 140,000 individual genes, of which 10-20% are expressed by the average cell (Dickson 1999, Liang & Pardee 1997). The genes expressed in a cell mirror the intracellular activities. With increasing knowledge of the gene sequence, there is a growing interest in finding the function of these genes. Several gene expression arrays are developing for large scale screening of differentially expressed genes (Matz & Lukyanov 1998). One such assay is the differential display of mRNA (Liang & Pardee 1992).

![Graph showing differential display footprint. All PCRs were run in duplicates and the differences in expression between the groups compared had to be present in both PCR footprints to be chosen for further studies. Arrows indicate differences.]

Figure 5. A typical differential display footprint. All PCRs were run in duplicates and the differences in expression between the groups compared had to be present in both PCR footprints to be chosen for further studies. Arrows indicate differences.
Differential display of mRNA includes three steps, reverse transcription, PCR and footprinting on a denaturing polyacrylamide gel (Zhang et al. 1998) (Figure 5). Reverse transcription (RT) was performed on two groups of pooled RNA, using 4 different anchored poly(dT)-primers, T₁₂MN (M = A, G and C; N = A, G, C, or T). The two RNA pools compared in the differential display assay were isolated from tissues treated in the best way to select for the physiological condition studied. The next step was amplification of the cDNA-pools with the same poly(dT)-primer as was used in the RT, in combination with an arbitrary decaprimer. Theoretically, all expressed genes would be compared using the combination of 20 decaprimer and 4 poly(dT)-primers (Liang and Pardee 1992, Bauer et al. 1993). The amplified gene products were then separated on a denaturing polyacrylamide gel and visualized using autoradiography, resulting in an almost identical footprint. However, some differences were detected. These differences indicated differential expression of a gene. To identify the gene products, the cDNA clones were excised and reamplified. Subsequent subcloning and sequencing resolved the identity of the gene products.

Many advantages have been attributed to differential display of mRNA in comparison to other transcription screening strategies (Liang & Pardee 1992, Watson & Margulies 1993). Such advantages are for instance the need of a small amount of total RNA (1-2 µg), the ability to compare multiple groups of mRNA, and the possibility to detect low mRNA transcript levels. Indeed, a large amount of the genes isolated using the differential display assay turned out to be below the detection limit when back hybridized on northern blots containing RNA isolated from tissue treated in the same way as the tissue used for differential display of mRNA. Of the 69 mRNAs found to be upregulated in rat ovaries with a large amount of apoptosis, 54 % were below detection limit using northern blot analysis (Paper I). In addition to ovarian tissue, differential display has also been used to isolate genes regulated in human adipose tissue pieces incubated in the presence or absence of GH (Table 5). The use of 60 primer combinations resulted in more than 300 differentially expressed genes. 53 genes were back-hybridized, of which 11 genes were confirmed to be differentially expressed using northern blot hybridization assay. 43 % of the hybridized cDNA clones from adipose tissue pieces were below detection limit. This is in agreement with previously reported numbers, reporting 40 % of the genes to be below detection (Liang et al. 1993). Variations in the number of genes below detection may be related to the different tissues or physiological conditions studied. Actually, around 11 000 mRNA species, expressed in a cell, are present at a low level of about 5-15 molecules per cell, and about 500 mRNA are expressed at an intermediate rate of 300 molecules per cell. Only around four mRNA species are expressed in more than 12 000 copies per cell (Alberts et al. 1994). This indicates the importance of finding a way to study also genes expressed...
at a low rate. However, using northern blot analysis, we were not able to confirm differential expression of these genes, since the expression was below detection.

During the last few years, differential display of mRNA has been extensively improved. The high incidence of “false positive clones” has long been a problem. Previous results have detected from 25 to as much as 50 % false positive clones (Utans et al. 1994, Liang et al. 1993, Wan et al. 1996). This is in agreement with our data, which displayed 39 and 36 % genes with no difference in expression or false positives in ovarian and adipose tissue, respectively (Paper I, Table 5). These results were achieved although the PCR was run in duplicates and extra care was taken not to cross contaminate during the excising of the cDNA clone from the polyacrylamide gel. The cause of this high degree of “false positives” may instead be the introduction of PCR-artifacts like e.g. miss-incorporations and amplification-errors. The PCR-artifacts have been suggested to be caused by the low amount of RNA and the low annealing temperature used in the differential display assay (Matz & Lukyanov 1998). Recent improvements, of the differential display technique, have therefore been performed to increase the annealing temperature in the PCR, and to increase the amount of RNA used. The annealing temperature has been increased by using arbitrary primers containing an extension of at least 10 bp. By doing this, the

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**Table 5. cDNA clones isolated using differential display of mRNA on adipose tissue.**
(Jalouli et al. Manuscript)

<table>
<thead>
<tr>
<th>Adipose tissue</th>
<th>Totally &gt; 300 cDNA</th>
</tr>
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<tr>
<td></td>
<td>53 northern analysis</td>
</tr>
<tr>
<td></td>
<td>11 -confirmed on northern</td>
</tr>
<tr>
<td></td>
<td>23 -below detection</td>
</tr>
<tr>
<td></td>
<td>19 -no difference in expression or false positives</td>
</tr>
<tr>
<td>7 upregulated in GH+cortisol group</td>
<td></td>
</tr>
<tr>
<td>4 upregulated in cortisol group</td>
<td></td>
</tr>
<tr>
<td>9 unknown</td>
<td></td>
</tr>
<tr>
<td>2 known: ribosomal proteins L26 and S23</td>
<td></td>
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first PCR cycle have to be performed at low temperature, but the following cycles are instead performed at about 60°C (Zhao et al. 1995, Diachenko et al. 1996).

In the differential display assay used here, poly(dT)-primers were used to discriminate for mRNA transcripts. However, this also limits the resulting cDNA-clone to be located to the 3'-poly-A end, a result which is also our experience (Paper I, III, IV). In most cases, this part of the mRNA is not translated, and is therefore not always included in the gene databank. In addition, a large amount of alternatively spliced gene products will not be found with differential display technique. An independently developed technique called RAP-PCR (RNA arbitrary primed PCR) (Welsh & McClelland 1990) has proven to be more efficient in selecting mRNA from the open reading frame. Using this technique, all mRNA is reverse transcribed using either a random primer or a poly(dT)-primer. In the PCR, an arbitrary decaprimmer is used in both directions of the PCR reaction, resulting in a product more upstream of the poly-A sequence than when using differential display of mRNA. A different technique is to use motif primers in the PCR, theoretically resulting in the isolation of cDNA templates encoding proteins with particular structural motifs (Hsu et al. 1993). These improvements have indeed resulted in a more efficient assay.

In the differential display assay, genes are studied that are transcriptionally regulated during a physiological event. It is therefore important to use tissue material and treatment of tissue relevant for the physiological event studied in the differential display of mRNA. Differential display of mRNA has been used extensively since it was first published (Liang & Pardee, 1992). However, most studies have been performed on cell cultures. The relevance of transcriptional regulation of a gene must however be determined for additional cellines and tissues to be trustworthy. In addition, many physiological events involve the whole tissue. For instance, estrogen production by the ovarian follicle needs both theca cells and granulosa cells. We have used whole tissue as fundamental material for the differential display assay (Paper I-IV). To find genes upregulated during apoptosis in the ovary, it was important to compare cells with a high abundance of apoptosis, with cells containing low abundance of apoptosis. To fulfill these criteria, the DES-apoptosis model was chosen, since hypophysectomy induces apoptosis in the ovaries, and estrogen, and FSH inhibits this induction of apoptosis. Previous studies on this model have shown high abundance and low abundance of apoptosis, respectively (Billig et al. 1993, 1994).

For the ovarian analysis of gene transcripts during apoptosis, additional work was performed to evaluate the true value of the genes during this process. Northern blot analysis was used to study the regulation of found genes in additional ovarian and a
prostate apoptosis model. Upregulation of a gene detected in more than one apoptosis model would be more likely to be involved in apoptosis (Figure 6). One of the models used was the PMS-in vivo apoptosis model. Earlier results have shown that prepubertal 27-28 day-old rats show a considerable amount of apoptotic follicles, while PMS inhibits this process (Billig et al. 1993, 1994). In this model, the whole ovary was also studied. However, in contrast to the DES in vivo model, apoptosis was inhibited. This can explain why only aldose reductase, of the genes studied, showed an increased expression. One could expect a different expression pattern during the inhibition of apoptosis, compared to before the onset of apoptosis. The inhibition of apoptosis by PMS may involve different pathways than the removal of estrogen (Cory 1998).

Another ovarian apoptosis model used was the in vitro model. This was the same model used when showing that transcription and translation was important for the process of apoptosis (Paper I). In this model granulosa cells isolated from PMS stimulated rats, underwent a spontaneous onset of apoptosis when cultured (Billig et al. 1994). However, none of the genes studied, were upregulated during apoptosis in this model. The reason for this could be related either to the time, place of expression or to the treatment of the cells. The genes upregulated in the in vivo models may be switched on for only a short time. This upregulation may therefore be missed during the in vitro assay. Alternatively, in the in vivo models, genes may be expressed in any of the cells included in the ovary. The genes detected to be upregulated in vivo might therefore be expressed in other cells then granulosa cells. The genes upregulated may also need other cells to be expressed. Finally, the expression may be related to the treatment of the cells, either related to apoptosis or

Figure 6. Circles represent mRNA transcripts present during apoptosis in different apoptotic models. An increased mRNA expression of a gene during apoptosis in more than one model, would be more likely to be involved in apoptosis.
not. Apoptosis in cultured rat granulosa cells may involve an alternative pathway of apoptosis (Cory 1998).

As many cellular events of apoptosis are general to most cells undergoing apoptosis, it was interesting to study the genes also in other tissues. We studied the expression of ARG candidates in rat prostate after castration induced apoptosis (Kyprianou & Isaacs 1988). Two of the ARG candidates, cytochrome b and aldose reductase, turned out to be more expressed after castration (Paper I and III). This may indicate that these genes are involved in a more general pathway of apoptosis. However, it can not be excluded that the genes are upregulated because of the treatment.

5.2 **Genes upregulated during apoptosis in the ovarian follicles**

5.2.1 **Two novel genes**

Using differential display of mRNA and subsequent back hybridization of the found clones, five genes were found to be more expressed in ovaries with a high abundance of apoptosis compared to ovaries with a low abundance. One of these genes, ARG-33, was not found in the NCBI gene databank. However, recently several EST clones were included in the gene databank (accession AI713775; AI763758; AI711454; AI230470; A1577353; AA997026). These EST clones, isolated from a Rattus norvegicus normalized atrium, embryo, or whole-eye library, showed high homology to ARG-33. The homology was 99-100 % over most of the sequence. However, ARG-33 was missing an internal base sequence of about 30 bp (bp 152-183). The reason for this may be a species or tissue specific alternative splicing product, or the missing sequence may have been lost during PCR. Interestingly, ARG-33 was detected only in brain and ovary of the tissues studied. This may indicate that ARG-33 is a tissue specific gene, possibly involved in ovarian apoptosis (Paper I).

Another gene, ARG-9, showed high homology to a non-coding part of neurofascin (Paper II). Neurofascin was originally found using ankyrin-coupled affinity columns (Davis &
Bennett 1993), and is part of the L1 family of neuronal cell adhesion molecules (CAMs), also including CHL1, L1-CAM, and Nr-CAM (Holm et al. 1996, Hortsch 1996). L1-type molecules are multipotent, and are involved in e.g. myelination, neuronal cell migration, neurite outgrowth. These CAMs binds to the cytoskeletal linker protein ankyrin, which anchors them to the submembranous actin-spectrin cytoskeleton (Davis et al. 1993, Davis & Bennett 1994). However, ARG-9 did not show the same tissue distribution as neurofascin (Moscoso & Sane 1995). These data were confirmed using the full-length coding sequence of neurofascin as probe for northern blot hybridization to different tissues. In addition, a 300 bp section of the full-length neurofascin coding sequence also did not hybridize to the tissues studied. These data indicated that ARG-9 and neurofascin are not identical, and can not be alternatively spliced products of the same gene, as is frequently the case in the chicken neurofascin gene (Hassel et al. 1997). Interestingly, ARG-9 is homologous to the non-coding 5'-end of neurofascin. Most genes isolated with differential display of mRNA were found in the 3’-poly-A end. However, the 5’-end of neurofascin contains a poly-A signal and a stop signal (Chen et al. 1995). These data in addition to the differences in tissue distribution indicates that the 5’-end of neurofascin instead is the 3’-end of a novel protein.

5.2.2 Cytochrome b

Cytochrome b proved to be transcriptionally upregulated using differential display and subsequent northern blot hybridization (Paper I). Cytochrome b originates from mitochondrial DNA and takes part in the respiratory chain’s bc-complex. Mitochondria are involved in many important cellular events, including metabolism, disease, aging and apoptosis (Boore 1999). During early evolution of eukaryotes, mitochondria were incorporated into the cell, allowing the cell to confront the increasing aerobic milieu (Vellai et al. 1998). The mitochondrial proteins, including cytochrome c and apoptosis inducing factor (AIF), have been shown to be released from the mitochondria into the cytoplasm, inducing apoptosis. However, neither cytochrome c nor AIF originates from mitochondrial DNA, but instead from nuclear DNA. Cytochrome c is transported to the mitochondria in an inactive form. An activating heme group is added to cytochrome c when it arrives in the mitochondria. The active form of cytochrome c
is never found in the cytoplasm, unless the cell is undergoing apoptosis (Rödel 1997). The mechanism of cytochrome b during apoptosis can only be speculative. However, the role of mitochondria during apoptosis is well established.

5.2.3 Aldose reductase

Using differential display analysis, aldose reductase was shown to be upregulated in vivo in ovaries with high compared to low abundance of apoptosis (Paper III). Aldose reductase was cloned in 1987 (Carper et al. 1987), and belongs to the NADPH-dependent aldo-keto reductase family (Carper et al. 1989). The aldo-keto reductase family is a large family of enzymes known to catalyze the reduction of aliphates, aromates as well as aldoses to the corresponding alcohols and polyols. Aldose reductase is also involved in glucose metabolism. It converts D-glucose into sorbitol in the polyol pathway (Hers 1956) (Figure 10). This is a minor pathway during normal conditions, but the flux through the polyol pathway is markedly increased under hyperglycemic and diabetic conditions, leading to an accumulation of sorbitol in the cells. This accumulation of sorbitol has been suggested to be the cause of diabetic complications such as cataract, retinopathy, and neuropathy. Aldose reductase has also been shown to be involved in osmoregulation in the kidneys. Sorbitol is one of the osmolytes balancing the osmotic pressure between extracellular NaCl and urine (Burg 1994).

Figure 10. Polyol pathway
The finding of aldose reductase mRNA upregulation during apoptosis in two models for ovarian apoptosis and one model for prostate apoptosis suggests a role for aldose reductase in apoptosis in these tissues. Indeed, previous studies have shown results that coincide with this hypothesis. For instance, galactosemia involves an accumulation of galactitol, a process involving aldose reductase, and is characterized e.g. by ovarian failure. Rats fed an excess galactose diet show a decrease in the number of ovulated oocytes and a delayed oocyte development. The concomitant treatment of the rats with sorbinil, an aldose reductase inhibitor, resulted in prevention of the qualitative and quantitative defects in the oocytes (Meyer et al. 1992). Another study showed that female aldose reductase transgenic mice were infertile or could not maintain a pregnancy, indicating an important role of aldose reductase in the ovary and in the placenta (Yamaoka et al. 1995). In addition to these results, aldose reductase has been shown to be identical to bovine testis 20α-hydroxysteroid dehydrogenase (Warren et al. 1993). This is however not the case for 20α-HSD in other tissues and species. 20α-HSD is involved in the reduction of progesterone to the inactive form, 20α-hydroxyprogesterone (Wiest 1959, Wilcox & Wiest 1966). Rat ovarian 20α-HSD activity originates from two different isozymes, called HSD1 and HSD2, of which only HSD 1 is cloned (Noda et al. 1992, Seong et al. 1992, Miura et al. 1994). HSD1 was not identical to aldose reductase. Others and we have also shown that aldose reductase is present in the granulosa cells in follicles of all developmental stages, including atretic follicles (Iwata et al. 1990). The peak levels of aldose reductase expression and activity on the morning of proestrus coincide with the peak levels of the number of atretic follicles (Iwata et al. 1996, Engle 1927, Hirshfield 1983). These data show that aldose reductase is active and may be part of apoptosis in the ovary eliminating atretic follicles.

The increased expression of aldose reductase detected in the prostate suggest a general function for aldose reductase in apoptosis, and indeed previous data performed in diabetic research point towards a possible relation between apoptosis in general and aldose reductase. In a number of studies aldose reductase inhibitors have been shown to prevent both diabetic peripheral nephropathy and retinopathy (Schoemaker 1994, Robison et al. 1989). Apoptosis has been suggested to be the mechanism behind both these complications (Mizutani et al. 1996, Ortiz et al. 1997).
In addition, recent data show that overexpression of aldose reductase in HIT cells, a β-celline, results in the induction of apoptosis. A redox imbalance was suggested to be the cause of this effect (Hamaoka et al. 1999).

The possible role of aldose reductase in ovarian apoptosis, and perhaps in apoptosis in general has not been functionally determined. However, circumstantial evidence points towards a role in the induction of apoptosis. Such pieces of evidence are for instance the presence of aldose reductase in atretic follicles undergoing apoptosis, the upregulation of aldose reductase in two in vivo models of apoptosis in the ovary, and in an in vivo prostate apoptosis model.

5.2.4 β-catenin

Figure 12. Figure showing the different physiological activities of β-catenin in cell-cell adhesion and

![Diagram of Wnt/wingless signaling](image)

Wnt/wingless signaling. (GSK3β = glycogen synthase kinase 3β, APC = adenomatous polyposis coli).

β-catenin was shown to be upregulated in ovaries with a high compared to a low abundance of apoptosis using differential display of mRNA (Paper IV). β-catenin is a 92 kD protein, sharing 80% homology with the armadillo protein found in Drosophila. They both contain 13 central arm-repeats, also found in e.g. plakoglobin and APC. The arm-repeat is a highly conserved 42 amino acid sequence, but the function is unknown. The amino-terminal domain of β-catenin contains sites for phosphorylation by GSK-3β, and binding of α-catenin. The function of the
carboxy-terminal domain of β-catenin is unknown, although it is essential for the Wnt/wingless-signaling in Drosophila (Peifer & Wieschaus, 1990). β-catenin is part of the cadherin-catenin complex (CCC), including E-cadherin, β-catenin, p120, and α-catenin (Figure 12). E-cadherin consists of an extracellular N-terminal, adhesive domain, a membrane spanning domain and a cytoplasmic domain, which functions as a binding site for catenins. α-catenin contains binding sites for talin, α-actinin, paxillin, and actin. When β-catenin is bound to E-cadherin, α-catenin can bind to the CCC, anchoring actin filaments to the complex, and forming the basis for adhesion between two cells. Cytoplasmic β-catenin also binds to adenomatous polyposis coli (APC) and the human homologue of the Drosophila wingless gene, Wnt-1. APC can complex with, and degrade β-catenin, while Wnt-1, on the other hand acts to increase the cytoplasmic β-catenin levels. APC and Wnt-1 serve to control the amount of free cytoplasmic β-catenin. Glycogen synthase kinase 3β (GSK3β) is induced by high levels of free β-catenin, and stabilizes the APC-β-catenin complex by phosphorylation of APC, which results in an increased degradation of β-catenin. In the presence of Wnt-1 signaling, GSK-3β is repressed in response to the activation of the phosphoprotein dishvelled (dsh), resulting in less phosphorylation of the APC-β-catenin complex, and less degradation of β-catenin. In response to Wnt-1 signaling the amount of catenins found in the nucleus is increased. This may suggest a possible signaling pathway of Wnt-1. β-catenin has been shown to bind the transcription factor LEF-1, a mammalian HMG box factor, which leads to the translocation of β-catenin to the nucleus. XTcf-3 similarly binds to β-catenin and translocates it to the nucleus (Miller & Moon 1996, Peifer 1997, Behrens et al. 1996, Molenaar et al. 1996). The β-catenin-Tcf/Lef complex then promotes transcription of e.g. E-cadherin, c-myc and cyclin-D1 (Huber et al. 1996, He et al. 1998, Tetsu & McCormick 1999).

Our data suggest a role for β-catenin in ovarian apoptosis. To test this we overexpressed β-catenin in CHO cells (Paper IV). The results turned out to be intriguing. The upregulation of β-catenin in ovaries with a high abundance of apoptosis detected using differential display of mRNA would suggest a role for β-catenin in the induction of apoptosis. However, β-catenin did not induce apoptosis when overexpressed in CHO cells. Other possible mechanisms for β-catenin during apoptosis would be either to enhance already induced apoptosis or to inhibit apoptosis. To test these possibilities we overexpressed β-catenin in CHO cells and subjected them to staurosporin, which is known to induce apoptosis in several
cellines. Surprisingly, the results showed that β-catenin inhibited staurosporin induced apoptosis in the CHO cells by 23 % after 5 h exposure. Bcl-2, which is a well-known inhibitor of apoptosis, was used as a reference for inhibition, and showed a 40 % inhibition at the same circumstances.

Indeed, β-catenin was recently found to be involved in apoptosis. The N-, and C-terminal domain of β-catenin were cleaved by caspases caspases-3 and -6, resulting in reduced α-catenin binding capacity. This further results in a release of actin-filaments reducing the cell-cell interaction (Herren 1998). This implicates that β-catenin is part of ovarian apoptosis, as loss of cell-cell interactions can be seen in apoptotic granulosa cells (Hsueh et al. 1994). However, this is not the only possible mechanism by which β-catenin may inhibit apoptosis. Several papers involving apoptosis, have shown proteins that take part in the Wnt/wingless signaling to interact with β-catenin. Secreted apoptosis-related proteins (SARPs), isolated using differential display of mRNA, have for instance been shown to modulate transcriptional levels of β-catenin. SARP1 transfected MCF7 cells were more resistant to apoptotic stimuli, while SARP2 transfected cells became more susceptible. These genes induced an increase and a decrease in β-catenin, respectively (Melnkonyan et al. 1997). Similarly, β-catenin is stabilized by the Alzheimer's associated protein presenilin-1. Mutations in presenilin-1 reduce this preserving effect, and results in an increased degradation of β-catenin and an increased susceptibility to apoptosis induced by amyloid-β protein. Inhibition of β-catenin signaling through Tcf-Lef resulted in an increased neuronal death induced by amyloid-β. These results were reversed by addition of either β-catenin or Tcf (Zhang et al. 1998). Our results in combination with previous results strongly suggest that β-catenin acts to inhibit apoptosis in the cells studied.

5.3 Apoptosis in the ovary - a possible mechanism for the cDNA clones.

The ovarian follicle includes different cells, which in a complicated system, regulated by hormones and paracrine/autocrine factors, proliferate, differentiate and undergo apoptosis (Chun & Hsueh 1998). The primordial and early antral follicles, which are not dependent on hormones, seem to be regulated by paracrine factors acting between the oocyte and the surrounding granulosa cells (Hsueh et al. 1996). The oocyte is also the first cell in these follicles to show any sign of apoptosis (Driancourt & Thuel 1998, Morita et al. 1999). However, the hormonally regulated
antral follicle, becomes eliminated in the absence of gonadotropins. The gonadotropins act via paracrine/autocrine factors, which induce apoptosis in the granulosa cells (Chun & Hsueh 1998). Apoptosis is induced also in the oocyte, but at a later stage (Tsafriri & Braw 1984). In addition, apoptosis is also induced in the theca cells, perhaps after they have phagocytosed remnants of the oocyte and the granulosa cells (Palumbo & Yeh 1994, Svensson et al. 1999). The intracellular mechanism by which apoptosis occur in the ovary seem to involve the basic elements. The Bcl-2 family members decide about pore opening in mitochondria and the release of cytochrome c and AIF. The caspases become activated and induce DNA degradation and proteolysis of structural proteins, resulting in condensation and formation of apoptotic bodies (Robles et al. 1999). Theca cells, expressing SRBI, attracts granulosa cells, possibly by recognizing phosphatidyl serine, which becomes externalized at a final stage of apoptosis (Svensson et al. 1999).

The role of the genes found using differential display analysis in the ovarian process of apoptosis can only be speculative. ARG-33, which was expressed only in brain and ovary, showed a clear-cut increase in ovarian mRNA expression as a response to estrogen withdrawal from hypophysectomized estrogen treated rats (Paper I). ARG-33 was not upregulated in other ovarian models of apoptosis, indicating a strong role of estrogen in the regulation of ARG-33. Indeed, several intraovarian factors are involved in the initiation of apoptosis (Chun & Hsueh 1998). Therefore, it is not contradictory to say that ARG-33 may induce apoptosis in the ovary as a result of withdrawal of the survival factor estrogen. It is also conceivable to say that the upregulation of ARG-33 can be an early preparation for apoptosis, as is the case for e.g. Apaf-1 and Bax (Robles et al. 1999, Kugu et al. 1998). However, to find out the specific mechanism by which ARG-33 may participate in ovarian apoptosis, the full sequence information is needed.

ARG-9 was found to be more expressed in ovaries with a high compared to a low abundance of apoptosis in two in vivo models for apoptosis in the ovary (Paper II). The absence of regulation in the in vitro model of apoptosis, could be explained by a possible expression in other cells but granulosa cells. Indeed, other studies have shown a lack of effect in cultured granulosa cells (Billig 1996). FSH, LH, GH and IGF-I, all known to inhibit apoptosis in cultured follicles, did not affect apoptosis in cultured granulosa cells. The possible explanation to this would be that they exert their effect via theca cells, possibly by other factors (Billig 1996). The intracellular mechanism for ARG-9 during apoptosis require full-length cloning as in the case of ARG-33.

Cytochrome-b is an interesting molecule, which was shown to be upregulated in apoptotic ovaries after estrogen withdrawal (Paper I). This molecule is located to the
mitochondria and takes part in respiration. Cytochrome c, which is another mitochondrial molecule taking part in the respiration, has been shown to be part of the general apoptotic mechanism. Pore opening in mitochondria usually results in the release of several molecules inducing apoptosis, e.g. cytochrome c and AIF (Motyl 1999). Cytochrome b would be a possible additional candidate for the induction of apoptotic events after a release through pore opening. However, cytochrome b is part of the bc-complex, and therefore not a free molecule situated in the intracellular space, like for instance cytochrome c and AIF. In addition, cytochrome b was not regulated in other ovarian models for apoptosis, indicating a strong relation of cytochrome b to withdrawal of estrogen (Paper I). It is therefore not likely that cytochrome b is released from mitochondria as a result of pore opening, since the release of cytochrome c is a more general mechanism of apoptosis, also involved in all models for apoptosis in the ovary. The effect of cytochrome b may instead be related to induction of apoptosis by e.g. reactive oxygen species (ROS) (Wong et al. 1989, Tilly & Tilly 1995).

Aldose reductase was shown to be upregulated in apoptotic tissues of three in vivo models but not in the in vitro model, indicating the importance of paracrine factors as regulators of aldose reductase expression (Paper III). Previous results have shown that aldose reductase can induce apoptosis in pancreatic cells, possibly by a redox imbalance in NADPH (Hamaoka et al. 1999). A redox imbalance could result in an intra-mitochondrial imbalance, generating pore opening (Hsueh et al. 1996). An aldose reductase caused redox imbalance would be a more general mechanism, involved in other tissues in addition to ovaries, since the mitochondria seem to be a central event of apoptosis. This could explain the upregulation of aldose reductase also in the prostate after castration induced apoptosis (Paper III). A more conceivable effect of aldose reductase in the hormonally regulated follicles, is however its' 20α-HSD activity (Warren et al. 1993). Progesterone is an important hormone released primarily from corpus luteum, taking part in embryo implantation, growth and development. Progesterone is also expressed by atretic follicles. It is possible that progesterone acts as a survival factor, as degeneration of progesterone by 20α-HSD in corpus luteum contributes to apoptosis of luteal cells (Stocco & Deis 1998, Yoshida et al. 1997). 20α-HSD inactivates progesterone by catalyzing the conversion to 20α-hydroxyprogesterone (Wiest 1959), and the peak levels of HSD 1 are at the end of the luteal phase. Aldose reductase, which was shown to be identical to bovine 20α-HSD, may act as a 20α-HSD also in the ovary, to inactivate progesterone and induce apoptosis in granulosa cells.

β-catenin was shown to inhibit staurosporin induced apoptosis in CHO cells, indicating that β-catenin may act as an inhibitor also in the ovary (Paper IV). Previous results support this theory (Melkonyan et al. 1997, Zhang et al. 1998). This
inhibitory role of β-catenin during apoptosis may be complex. Upregulation of β-catenin was only present in ovaries from hypophysectomized rats after estrogen withdrawal. The upregulation detected in this model may therefore be a result of a preventative action to inhibit apoptosis, tightly coupled to estrogen withdrawal. Bax and Apaf-1 have been shown to be upregulated prior to apoptosis in the ovary, perhaps as a preparation for apoptosis (Robles et al. 1999, Kugu et al. 1998). In the PMS in vivo apoptosis model, apoptosis is instead inhibited, which can explain the lack of upregulation in this model (Tilly et al. 1992a). The lack of β-catenin regulation in the in vitro apoptosis model, suggests that additional cells are needed to regulate β-catenin expression.

The proteolytic cleavage of β-catenin by downstream caspases indicates that β-catenin has an important structural role, at a late stage of apoptosis (Herren et al. 1998). Previous results have shown the importance of cell-cell interactions for granulosa cell survival (Hsueh et al. 1994). Indeed, recent data suggests that functional N-cadherin is necessary for the survival of granulosa cells. Granulosa cells undergoing apoptosis displayed a decrease in N-cadherin (Makrigiannakis et al. 1999). In addition to being part of cell-cell adhesion, β-catenin is also a transcription factor (Huber et al. 1996, He et al. 1998, Tetsu & McCormick 1999). Interestingly, β-catenin induces transcription of e.g. E-cadherin, which is part of the cell adhesion complex. Upregulation of other molecules may also contribute to the inhibition of apoptosis.
6. SUMMARY and CONCLUSIONS

Apoptosis is a physiological event, which in a neat and tidy way eliminates a defect cell, or a cell, which is no longer needed. The intracellular events associated with apoptosis are currently under investigation. Common events involve the initiation, decision making, execution and phagocytosis. Most of these steps involve post transcriptional regulation. However, hormonally dependent apoptosis has, in many cases, been shown to involve transcriptional regulation.

In this thesis, evidence are presented showing that apoptosis, spontaneously induced in granulosa cells, was dose-dependently inhibited by the transcription and translation inhibitors, actinomycin-D and cycloheximide (Paper I). These results indicate that apoptosis in granulosa cells in vitro and possibly also in vivo is dependent on transcription and translation.

Differential display of mRNA was evaluated in this thesis. The assay was performed on ovarian RNA isolated from hypophysectomized rats with estrogen implants or two days after withdrawal of implants, resulting in low and high abundance of apoptosis, respectively. Back hybridization of cDNA clones found using differential display of mRNA resulted in 7% confirmed transcriptionally upregulated genes (Paper I). 54% of the genes were below detection limit in the northern blot assay and 39% of the genes showed either no difference in expression or were even false positives. These data prove differential display of mRNA to be sensitive, as it detects several genes that can not be confirmed with northern blot technique. It is also sensitive to differences that may be of individual character or to false positive clones, since the differences could not always be repeated using northern blot analysis. Several improvements have been made to the differential display technique, during the last few years, which may result in a better screening of cellular transcripts.

Using differential display of mRNA five genes were shown to be upregulated in ovaries with a high abundance of apoptosis, compared to a low abundance of apoptosis (Paper I). Two were novel genes, dedicated ARG-33 and ARG-9 (Papers I and II). Cytochrome b, aldose reductase and β-catenin also showed higher expression in ovaries with more apoptosis (Papers I, III and IV). ARG-33, ARG-9, cytochrome b and aldose reductase may be related to apoptosis or they may be related to the treatment. Interestingly, β-catenin was shown to inhibit staurosporin
induced apoptosis in cultured CHO-cells, indicating that this protein may be an apoptotic survival factor also in the ovary.
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