REGULATION OF CELL-CELL ADHESION AND TRANSCRIPTION IN THE OVARY:
IMPLICATIONS FOR TUMORIGENESIS

Karin Sundfeldt
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Karin Sundfeldt, Department of physiology and pharmacology, section of endocrinology, University of Göteborg, Sweden

ABSTRACT

In this thesis the expression and localization of the epithelial (E)-cadherin/α - and β -catenin complex was investigated during the normal ovarian cycle and in ovarian tumors of epithelial origin. Furthermore, transcription factors (TFs) such as the proto-oncogene protein c-Myc was investigated for its time- and cell-specific expression during follicular development and the expression of CCAAT/Enhancer binding proteins (C/EBPs) were investigated in ovarian tumors of epithelial origin.

The development of ovarian follicles is a continuous process. Cyclic stimulation by hormones and growth factors will lead to proliferation and differentiation of granulosa cells (GCs) and theca cells (TCs). This follicular development also involves dramatic changes in the cell’s morphology. Cells are held together by junctions i.e. tight-, adherens- (AJ) and gap-junctions. AJs, which are build up by the cadherin/catenin complex, are present between GCs of primary follicles but disappears in the fully developed preovulatory follicle. Regulation of E-cadherin at the transcriptional level could be managed by TFs such as c-Myc, C/EBPs and AP2.

The ovarian surface epithelium (OSE) is the origin for approximately 90% of the malignant ovarian tumors. The tumors arise from the inclusioncysts, localized in the ovarian stroma, and
grow solid, cystic or in mixed formations. Intra-abdominal spread of the ovarian cancer is common and this is a process closely connected with impaired cell-cell adhesion.

In a rat model, where immature rats were stimulated by gonadotropins, normal development of follicles could be studied. Small primary follicles were found with positive E-cadherin and catenin staining. During proliferation and differentiation of the follicle, E-cadherin was only found in TCs and not in GCs, while the catenins were expressed in both celltypes. Just prior to ovulation, the staining of E-cadherin/catenin complex was decreased in the TCs.

The E-cadherin protein was not found in OSE of normal ovaries but in inclusion cysts, as well as in benign and malignant ovarian tumors. The soluble form of E-cadherin was found in peripheral blood, cystic- and ascitic fluid. The concentrations were significantly increased in cystic fluids from patients with borderline and malignant tumors when compared to patients with benign adenomas.

With a similar approach, as described for E-cadherin, the time and cell-specific expression of c-Myc mRNA and protein was analyzed in rat ovaries. The levels of c-Myc were found to be transiently elevated during the development of the preovulatory follicle. The LH-surge resulted in a rapid increase in c-Myc mRNA (after 1h) and c-Myc protein (after 2h). The change in c-Myc expression was mainly localized to the GCs.

The expression of C/EBPs (α, β, δ and ζ) were mainly seen in the epithelial cells of the normal human ovary and ovarian tumors. The most prominent finding in this study was the increased expression of C/EBPβ in the nucleus of cells in ovarian adenocarcinoma as compared to the benign adenoma and the normal ovary.

**Key words:** E-cadherin, catenin, ovary, follicle development, ovarian surface epithelium, ovarian neoplasm, cystic fluid, proto-oncogene protein c-Myc, CCAAT/enhancer binding protein (C/EBP), cancer

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THE OVARY

OVARIAN EMBRYOLOGY AND ANATOMY

The gonads (ovary and testis) are formed from the mesodermic cell layer during fetal development. These tissues initially appear as genital ridges, which are formed by proliferation of the coelomic epithelium and condensation of the underlying mesenchyme. The coelomic epithelium will then penetrate into the mesenchyme and form cortical cords, which break into clusters with primitive germ cells (oogonia) close to the surface epithelium. Each germ cell will by this mechanism be surrounded by epithelial cells, which are descendants of the surface epithelium. These cells will later form the follicular cells of the primordial follicle.

The outermost layer of the ovarian cortex is a single germinal epithelium, which is commonly referred to as the ovarian surface epithelium (OSE). These cells have either a flat or a cuboidal appearance. This epithelium is often referred to as a mesothelial type of epithelial cells, since they share embryological background and some characteristics with the mesothelium, which is lining the peritoneal cavity. Beneath this cell layer, the tunica albuginea, a poorly delineated layer of dense connective tissue, is situated. This stroma like layer consists of connective tissue with fibroblasts, smooth muscle cells, endothelial cell and interstitial cells including undifferentiated theca cells (TCs), degenerated follicular cells from atretic follicles or regressed corpora lutea.

OVARIAN PHYSIOLOGY AND HORMONAL REGULATION

At the time of birth, the ovary contains small primordial follicles consisting of the oocyte and a single layer of flat epithelial/follicular cells. These follicles will rest in this position until the onset of puberty. Under the influence of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), these immature follicles will develop into primary follicles. The flat epithelial cells of the follicle proliferate and differentiate into granulosa cells (GCs) with steroidogenic capacity. In the preovulatory (Graafian) follicle, the antrum and TC layers (interna and externa) are distinct structures. Theca cells also acquire steroid producing functions. Since the blood and nerve supply to the follicle end in the theca interna a "blood-follicle barrier" between GCs and TCs has been suggested. The communication between GC-GC and GC-oocyte is maintained mainly through gap junctions.
The preovulatory (Graafian) follicle undergoes extensive structural and functional changes in response to LH. The results of this LH-surge are maturation of the oocyte, ovulation and formation of a corpus luteum (luteinization). The non-ovulating large antral follicles will become atretic, a process characterized by apoptosis (Hsueh et al. 1996). Blood vessels will in response to the LH-surge become leaky and the thecal tissue will become edematous. Fibroblasts in the stroma layers will proliferate, dissociate and take on a looser connective tissue-like appearance. Specific changes take place in the perifollicular tissue underlining the OSE i.e. focal reduction in tissue perfusion (Brannstrom et al. 1998) and inflammation-like events (Espey 1980). The cells of the OSE accumulate lysosomes and during these processes these cells may secrete proteolytic enzymes responsible for OSE degradation (Bjersing et al. 1974a, 1974b). The ovulation process, then leads to fragmentation and flattening of the OSE and accumulation of fluid between the cells, which break apart and retract away from the expulsion area (Van Blerkom et al. 1978) (Nicosia et al. 1991).

After the onset of the LH-surge, at the same time as the oocyte undergo maturation changes and the follicle prepares for rupture, the GCs and TCs start to proliferate and differentiate into luteinized cells. This also involves vascularization of the previously avascular GC-layer facilitated by degeneration of the GC-TC barrier. During this luteinization process the mature and the progesterone producing corpus luteum is generated.

**GENE EXPRESSION IN THE OVARY**

Gonadotropins and locally produced growth factors bind to specific receptors on the follicular cells. This ligand-receptor bindings activate intracellular signaling pathways i.e. the protein kinase A (PKA)-pathway with subsequent increases/decreases in transcription of the targeted gene (Richards et al. 1995). Different genes are expressed in a highly regulated and complex fashion during the events of follicular development, ovulation, corpus luteum formation, and corpus luteum demise (luteolysis). The gene expression is further customized to the specific cells of the ovary. In general, most knowledge in this field comes from studies of the GCs (Richards 1994).

There are now several well described transcription factors that are regulated by gonadotropins in the ovary. In this thesis I will more specifically discuss the proto-oncogene protein C-myc, of the helix-loop-helix-leucin zipper family, and CCAAT/enhancer binding protein (C/EBP), of the leucin zipper family. Their specific expression patterns/regulation in the ovary and ovarian tumors as well as possible target genes will be presented in the chapter entitled specific background.

**CELL ADHESION**
INTRODUCTION

During embryonic development, adhesive interactions play mayor roles in the triggering of a variety of morphogenic processes. Such interactions may involve either direct cell-cell contact or adhesion to extra-cellular matrix. The knowledge in this area has exploded since the first cell-cell adhesion molecule (CAM) was discovered 20 years ago (Edelman 1976, Thiery et al. 1977). In Figure 2, an overview of junctional mechanisms in epithelial cells is given.

Figure 2. Overview of junctions and their corresponding cell-cell adhesion molecules.

The adherens junction consists of a cell membrane-bound protein from the cadherin family of CAMs. This type of junction is primarily found in plaque formations and adheres to other cells in a homophilic way. The intracellular tail binds cytoplasmic proteins. These cytoplasmic proteins belong to the family of catenins, which stabilizes the complex by connection to the cytoskeleton. Details about the cadherin/catenin complex will be presented in the chapter entitled specific background.

The formation of adherens junctions might be an important mechanism to provide mechanical strength and to provide polarization and orientation of the cells within any organized tissue, while the non-junctional adhesion might promote locomotion of cells. The list of mechanisms that are suggested to involve cadherin-mediated cell-cell adhesion is constantly growing. Today condensation and compaction of cells during embryogenesis, contact inhibition of cell growth, wound healing and tumorigenesis are some of the suggested processes in which cadherins have been suggested to be important. In this thesis I will focus on the role for the cadherin/catenin-complex during tumorigenesis.

CELL ADHESION DURING FOLLICULAR DEVELOPMENT

In the 1970´s Albertini et al (1974), by the use of freeze fraction analysis investigated follicular development and aspects of cell junctions. They found that during the early stages
of follicular development (primordial and primary follicles), contacts between GCs are characterized by adherens junctions, desmosomes and small gap junctions. Granulosa cells of these follicles are also small and tightly packed. As the follicle grows, gap junctions will increase in size and numbers while adherens junctions will decrease (Albertini et al. 1974, Amsterdam et al. 1992). The GCs of preovulatory follicles resemble mesenchymal cells and are more loosely attached (Albertini et al. 1975). The increase of gap junctions in GCs after stimulation by gonadotropins and/or estrogen was later established, in contrast to the whereabouts concerning changes in adherens junctions (Amsterdam et al. 1992, Grazul Bilska et al. 1997). The group of Amsterdam et al (Ben Ze'ev et al. 1987) took on another approach by studying the intermediate filaments (i.e. actin and vimentin) connected to the adherens junctions. They found that the presence of actin decreased after stimulation by gonadotropins, at the same time as steroidogenesis increased in cultured GCs. The importance of junctional complexes in follicular development has recently become evident when it was found that connexin 37 -/- mice do not develop tertiary or Graafian follicles and do not ovulate (Simon et al. 1997). The follicles of these mice lacked any type of gap junction, but in contrast to follicles of normal mice they retained their adherens junctions.

TUMORS OF THE OVARY

INTRODUCTION

The development of a tumor is the result of a series of events. Several mutations and a natural selection process for the more competitive cell population lead to an uncontrolled proliferation of cells and a tumor is formed. Additional changes in oncogenes and/or tumor suppressor genes, lead to loss of cell-cell adhesion, production of proteolytic enzymes and metastasis through direct invasion or transport of the tumor cells through the lymphatic ducts or blood vessels.

EPIDEMIOLOGY AND ETHIOLOGY

The epithelial-derived adenomas and adenocarcinomas account for 90% of the ovarian tumors. In Scandinavia, the lifetime risk for females to develop ovarian cancer is 1-2%, which is the highest lifetime risk for ovarian cancer in the world. In contrast, developing countries and developed countries with a different lifestyle such as Japan has less than 0.5% risk (Katso et al. 1997, Riman et al. 1998). The cause of ovarian cancer is poorly understood but some theories with support from both epidemiological and cellbiological studies have been put forward. The "incessant ovulation" theory (Fathalla 1971) hypothesis that the overall risk for developing ovarian cancer is related to the number of ovulations that has taken place. The epidemiological facts gathered, states that events or interventions that decrease the number of ovulations, such as childbirth, lactation and use of combined (estrogen/gestagen) contraceptives, lower the risk to develop epithelial ovarian tumors (Adami et al. 1994). Animals do not in general develop epithelial ovarian tumors, with the exception of hens that interestingly also have very high ovulation rates.

The "gonadotropin" theory is based on the fact that ovarian tumors in general develop postmenopausally when the blood levels of gonadotropins are markedly elevated, which results from the loss of negative steroidogenic feed-back. Most epidemiological data speaks against this latter hypothesis, but the biological theories behind these hypotheses can serve as
explanations for each or both. Ovarian tumors often contain gonadotropin receptors and ovarian cancer cells respond to gonadotropins with proliferation (Elbendary et al. 1996, Riman et al. 1998). At the time of ovulation, the OSE breaks apart and the cells will then become exposed to extremely high levels of estrogens and gonadotropins in the follicular fluid, which penetrates through the mesh-like exterior follicular wall during several hours before actual rupture (Zachrisson 1997). The repetitive proliferation of OSE, to cover the apex defect after ovulation, may increase the frequency and accumulation of spontaneous cancer-associated mutations.

Finally, there is the entrapment of OSE in the stroma of the ovary, which is the beginning of the inclusion cysts (Nicosia et al. 1991, Hamilton 1992, Scully 1995).

HISTO-PATHOLOGY

Epithelial-derived ovarian tumors develop from inclusion cysts in the stroma. Tumors can be grouped into benign adenomas, borderline tumors and malignant tumors (adenocarcinomas). There are several histopathological subgroups such as, serous (most common), mucinous, endometroid, clear cell and mixed or undifferentiated adenocarcinoma. The malignant tumors grow solid or in cyst formations and will typically invade the surrounding lower abdomen, set metastasis in the peritoneal cavity by preference and more seldom give rise to peripheral metastasis. The tumors are surgically staged (I-IV) and then graded (highly, moderately, poorly, or un-differentiated) according to the classification of the International Federation of Gynecology and Obstetrics (FIGO).

MOLECULAR BIOLOGY

It is generally believed that ovarian cancers, like most other cancers, arise due to the mutations or rearrangements of oncogenes and tumor suppressor genes. These genes are normally involved in the regulation of cellular proliferation, differentiation and apoptosis. Inherited ovarian cancer (approximately 5-10%) have especially been connected to mutations of the breast tumor related antigen (BRCA-1) tumor suppressor gene, since BRCA-1 carriers have a 63% risk of developing ovarian cancer. The function for the BRCA-1 gene product is still unknown. The majority of epithelial ovarian tumors are sporadic cancers and genes i.e. epidermal growth factor receptor (EGFr), Her-2/neu receptor tyrosine kinase, ras family of G proteins, transcription factor c-Myc, retinoblastoma (Rb) and p53 (Boente et al. 1993, Berchuck et al. 1997) are known to be connected to the development and progression of this tumor. However, gene analysis shows a very heterogeneous picture with changes in the ovarian tumor DNA at several locations and with great difference in-between patients.

The mechanism of metastasis development of ovarian cancer from the ovary to the peritoneum is difficult to study mainly due to that suitable experimental models are lacking. One approach is to investigate the expression of genes and proteins involved in cell-cell, cell-matrix adhesion and matrix degrading functions.

SPECIFIC BACKGROUND

In this section there will be a description of cell-cell adhesion molecules involved in normal ovarian cell adhesion and ovarian tumors with specific focus on the cadherin/catenin complex. Furthermore, the transcription factor families of c-Myc and CCAAT/enhancer binding protein (C/EBP) will be introduced in general and also in an ovarian-related point of view.
THE EPITHELIAL (E)-CADHERIN, α - AND β -CATENIN - COMPLEX

INTRODUCTION

Cadherins belong to a family of single transmembrane, Ca²⁺-dependent, homotypic binding, cell-cell adhesion proteins. E-cadherin was discovered in the beginning of the 1980’s and has been known under many names; Arc-1 (Imhof et al. 1983), uvomorulin (Urushihara et al. 1980), L-CAM (Brackenbury et al. 1981), cell-CAM 120/80 (Damsky et al. 1983). The locus for human E-cadherin has been mapped to 16q22.1. E-cadherin is synthesized from a 4.5-kb mRNA as a 135-kDa precursor polypeptide, which is processed within 2h by proteolytic cleavage to the mature 120 kDa form (Shore et al. 1991). The extracellular part of 80 kDa can later be proteolytically cleaved (Damsky et al. 1983, Wheelock et al. 1987, Takeichi 1988). The classical cadherins are neuronal (N)-, placental (P)- and E-cadherin, which are all well characterized. However, there has been a growing number of family members in recent years and all vertebrate cells seems to express one or more cadherin (Takeichi 1991). The cytoplasmic tail is highly conserved and forms complexes with the catenins (see below). These proteins will in turn stabilize the cell-cell contact by binding to the cytoskeleton via actin (Geiger et al. 1992, Gumbiner 1996, Yap 1998).

It has been known for a long time that loss of epithelial differentiation in carcinomas, a type of epithelial/mesenchymal conversion, is related to a more aggressive and invasive tumor type. This conversion involves loss of functional cadherin adhesion in normal epithelium and down-regulation of E-cadherin in tumor cells (Van Roy et al. 1992, Birchmeier et al. 1994a). However, recently the ability of E-cadherin to induce desmosome formation and stratified epithelium in corneal fibroblast cells (Vanderburg et al. 1996, Tlsty 1998), a process of mesenchymal/epithelial conversion was described. The expression of E-cadherin correlated with epithelial differentiation and invasive state of some carcinomas. Other studies have shown intact E-cadherin in invasive cells, where either of the catenins was affected, as possible explanations for reduced cell-adhesiveness (discussed in detail in the section named General Discussion). Even though the roles for the E-cadherin/catenin complex in tumor invasion has been thoroughly investigated, the first studies which clearly show that E-cadherin loss actually leads to tumor formation or proliferation has recently been published (Guilford et al. 1998, Perl et al. 1998).
Figure 3.

Model of the E-cadherin/catenin complex and the Wnt/wingless signaling pathway

The catenins were first identified as a set of three (α, β and γ) proteins that would immunoprecipitate with E-cadherin (Gumbiner et al. 1993, Hinck et al. 1994, Yap 1998). Beta-catenin is known to bind directly to the cadherin cytoplasmic tail while α-catenin binds to β-catenin and connects the complex to actin or actinin. Cells lacking α-catenin are unable to form stable contacts despite high expression levels of E-cadherin and β-catenin. It is now well established that β-catenin also interacts with other cytoplasmic proteins (BenZeev et al. 1998). Thus, β-catenin has a role in the Wnt-wingless pathway, where stable complexes that facilitate degradation of β-catenin, are made with adenomatous polyposis coli (APC) gene product and glycogen synthase kinase-3β (GSK3β) in normal cells (Rubinfeld et al. 1996) (Peifer 1996). In certain cancer cell lines with mutational changes in these proteins, a free pool of β-catenin is found and can bind to the transcription factor T-cell factor/Lymphocyte enhancer factor (Tcf/Lef-1) (Korinek et al. 1997, Morin et al. 1997, Rubinfeld et al. 1997) (Peifer 1997). Together they translocate to the cell nucleus (Behrens et al. 1996, Molenaar et al. 1996) and can initiate transcription of i.e. the c-Myc, E-cadherin and cylin D1 genes (Huber et al. 1996b, He et al. 1998, Tetsu et al. 1999). It is also of interest that catenins are present in cells with little or no cadherin (Geiger et al. 1992).

THE CADHERIN/α- AND β-CATENIN – COMPLEX IN THE OVARY

In this section I will only present the findings in this particular area which had been published up to the time of the start of this PhD project. Thus, this was the background of papers I-III. More recent findings will be brought up in the general discussion.

In 1989, it was shown that GCs from estrogen (DES)-primed animals expressed E-cadherin protein (Farookhi and Blaschuk 1989). This observation was followed by a study who showed an increase of E-cadherin mRNA in whole ovaries of immature mice after systemic injection of estradiol (E2) (MacCalman et al. 1994). Furthermore, E-cadherin expression was examined in a murine ovarian tumor cell line (OV2944) with sublines of different metastatic activity (Hashimoto et al. 1989). Northern blot analysis showed high levels of E-cadherin mRNA in the cell lines with low metastatic potential compared to low levels in cell lines with high metastatic capacity. However, then it was found that E-cadherin was expressed in 9 out of 9 immunohistochemically stained tumors of the human ovary (Inoue et al. 1991). In fact, in mutational studies of the E-cadherin gene in ovarian cancers (n=63), using single strand
conformation polymorphism (SSCP) technique, only one of the investigated ovarian tumors exhibited a mutation (Risinger et al. 1994).

N-cadherin had been described in cultured rat GCs and the expression increased after stimulation by E2. Addition of FSH did not give any further increase and FSH alone had no effect on N-cadherin expression (Blaschuk et al. 1989, Farookhi and Blaschuk 1991).

OTHER CELL-CELL ADHESION MOLECULES IN THE OVARY

THE NEURAL CELL ADHESION MOLECULE (NCAM)

A couple of studies have investigated the expression of NCAM mRNA and protein in the ovary (Mayerhofer et al. 1991, 1994, Moller et al. 1991). The NCAM family consists of three major members generated by alternative splicing of a single gene (180 kDa, 140 kDa and 120 kDa) (Edelman et al. 1991). Only the two smaller proteins are expressed in the rat ovary. By immunohistochemistry, NCAM was localized to the GCs and the luteal cells of ovaries from both rat and mouse (Mayerhofer et al. 1991). Granulosa cells from preovulatory follicles of the human ovary and cultured GCs that underwent luteinization in vitro, both expressed NCAM-140 mRNA and protein (Mayerhofer et al. 1994). The authors conclude from these studies that NCAM may be involved in corpus luteum formation. It is interesting to note that NCAM-140 has been reported to downregulate the expression of matrix metalloproteinases (MMPs) i.e. interstitial collagenase (MMP-1) and 92-kDa gelatinase (MMP-9) (Edvardsen et al. 1993). MMPs are produced in the rat ovary and MMP-1 in particular is proposed to be important in ovulation (Reich et al. 1991, Tadakuma et al. 1993). In ovarian carcinoma, NCAM staining was seen in 7 out of 28 tumors that were investigated (Kaufmann et al. 1997).

INTEGRINS

The members of the cell-adhesion family of integrins build up cell-matrix connections and act as receptors for extracellular matrix proteins. This family of CAMs consists of several members built up by different α and β chains. The mouse ovary was examined for the expression of integrin α 6 (Fröjdman et al. 1995). This integrin was expressed in the ovary during embryogenesis and in adult mice α 6 was present in the OSE and in TCs of the larger follicles. Several groups have found, that in particular integrin α 6 but also α 2, α 3 and β 1 are expressed during follicular development and corpus luteum formation in time- and cell-specific manners, indicating important roles for the integrins during these processes (Aten et al. 1995, Honda et al. 1995, Giebel et al. 1996, Nakamura et al. 1997, Fujiwara et al. 1998).

There is clearly a role for integrins in cancer development or growth. Various tumor types stop producing specific integrins, display new types of integrins or alter the distribution of integrins. These alterations are thought to facilitate migration of the tumor cells (Horwitz 1997). In the human ovary, expression of integrins α 1, α 3, α 6 and β 4 were observed in the
OSE (Bartolazzi et al. 1993, Skubitz et al. 1996). In malignant primary tumors, loss of integrin α 1 was found (Bridges et al. 1995, Liapis et al. 1997, Buczek Thomas et al. 1998). In ascitic tumor cells, integrin β 4 and α 6 are downregulated in contrast to high expression of these integrins in both normal OSE and primary tumors. Defined functions for integrins specifically in ovarian tumor progression or invasion have not been described.

GENE TRANSCRIPTION AND REGULATION

A transcription factor, a set of transcription factors, enhancers or control elements bind to the promoter region of the targeted gene in a specific way so that transcription can start. The transcriptional control is quite complicated and the more crucial the transcript is for the survival of the cell, the more complex is the control machinery in relation to the gene-transcription at the DNA level.

The transcription factors are grouped into different families depending on the way they bind to DNA. Transcriptional regulation and control of the E-cadherin gene was, based on results of early promoter studies, suggested to involve an E-box, a CCAAT-box and a GC-rich region as possible DNA-binding sites (Hennig et al. 1996). The myc family was suggested to bind to the E-box consensus site and the C/EBP-family was proposed to bind to the CCAAT-box. It was later found that the CCAAT-box represents only weak C/EBP binding sites. Still, these families of transcription factors are both very interesting in the aspect of cell-proliferation and cell-differentiation and we were intrigued of the finding that also cell-cell adhesion might be related to these events.

THE PROTO-ONCOGENE C-MYC

INTRODUCTION

The c-Myc gene is very well preserved throughout evolution from Xenopus to humans. The gene codes for an mRNA of approx. 2.2 to 2.4 kb, which translate into proteins of 64 and 67 kDa. The c-Myc protein is a transcription factor of the basic helix-loop-helix-leucin zipper (bHLH Zip) family (Marcu et al. 1992, Zörnig et al. 1996, Bouchard et al. 1998). The partner protein of c-Myc in vivo is another bHLH Zip called Max. Dimerization with Max is required for DNA binding and transcriptional activation. The Myc-Max heterodimers bind mainly to the DNA consensus sequence CACGTG, also referred to as the "E-box" (Grandori et al. 1996).

In the normal cell, c-Myc expression is tightly regulated in response to growth signals. Non-proliferating, quiescent cells express low or undetectable levels of c-Myc but this expression is rapidly induced following mitotic stimulation. When the mitotic stimulus is withdrawn, c-Myc protein rapidly disappears. Both the mRNA and the protein are short lived, with t½ of about 30 min (Dean et al. 1986, Waters et al. 1991). C-Myc is also a potent inducer of apoptosis (Thompson 1998). Both reductions of c-Myc and inappropriate overexpression can be associated with cellular apoptosis. In human malignancies, c-Myc mutations that cause structurally normal or certain mutant forms of c-Myc to be expressed continually are found or are at levels even higher than those of normal cells. This overexpression is probably part of the multistep
oncogenic process and is often found in association with mutations of other proto-oncogenes e.g. ras.

The earliest response of activation of c-Myc is a rapid induction of cyclin-E-Cdk2 kinase activity. One potential target gene of c-Myc implicated in this activation is Cdc25A, a cell-cycle regulatory protein. This is a G1-specific protein phosphatase that is required for the initiation of the cell cycle and for its progression through G1 (Galaktionov et al. 1996). Other known target genes for c-Myc are, Cyclin A, Cyclin E, Cyclin D1, estrogen receptor, p53, C/EBPα, and E-cadherin (Leone et al. 1997, Facchini et al. 1998). Regulation of c-Myc is primarily on the mRNA and protein level by controlling their stability and regulation of their half-lives (Marcu et al. 1992) but transcriptional regulation has also been observed (Facchini et al. 1998).

C-MYC IN THE OVARY

In the rat ovary, c-Myc mRNA and protein rapidly increase in GCs after gonadotropin-priming of the immature rat (Delidow et al. 1990). This increase most likely reflects proliferation of the cells since, H³thymidine incorporation increase in the stimulated cells. Peri-fusion of immature ovaries treated with FSH and insulin shows the same type of short-time increase of c-Myc (Delidow et al. 1992). In other endocrine organs, such as the uterus, estrogen stimulates expression of c-Myc in the rat (Huet-Hudson et al. 1989) while progesterone lowers the oviduct concentration of c-Myc in the sheep (Fink et al. 1988).

In ovarian tumors, the c-Myc gene is amplified in 30-50% of the tumors (Zhou et al. 1988, Sasano et al. 1990) and the protein is simultaneously overexpressed (Bauknecht et al. 1990, Sasano et al. 1992). In one report, it was demonstrated that the levels of c-Myc protein increase prior to development of aneuploidy during the progression of the ovarian malignant phenotype (Watson et al. 1987). In an ovarian cancer cell line (OVCAR3), derived from a moderately differentiated serous adenocarcinoma, estrogen stimulated the expression of c-Myc with subsequent proliferation of the cancer cells (Chien et al. 1994). In the same study treatment with antisense c-Myc abolished proliferation of the estrogen stimulated cells.

Recently the c-Myc mRNA levels was investigated to assess whether they were related to ovarian tumor expression of estrogen receptors, metastasis, survival time, FIGO stage, histological grade and type. Approximately 30% of the 90 tumors contained detectable levels of c-Myc mRNA but there was no correlation with any of the investigated parameters (Tanner et al. 1998).

CCAAT/ENHANCER BINDING PROTEIN (C/EBP)

INTRODUCTION

To date, six members of the C/EBP family of transcription factors have been characterized. According to the nomenclature suggested by Cao et al (1991), these members are called C/EBPα, β, δ, ε, γ and ζ. Similarities between the C/EBP family members suggest a common evolutionary history of the gene, even though the different family members vary in tissue specificity and transactivating ability (Lekstrom Himes et al. 1998). Moreover, all of them make homo- as well as hetero-dimers with each other (Descombes et al. 1990, Cao et al. 1991, Ron et al. 1992) in order to activate transcription or direct the complex away from the DNA-binding site. There have also been descriptions of protein-protein interactions between
C/EBP-family members and NF-κB proteins, p21 and Rb (LeClair et al. 1992, Matsusaka et al. 1993, Stein et al. 1993, Chen et al. 1996b, Timchenko et al. 1996). Interactions with the latter two are pointing towards possible regulating functions during the cell-cycle progression.

The first member of the C/EBP family which was cloned (C/EBPα), was identified as a protein binding to CCAAT-boxes (Landschulz et al. 1988). However, the optimal binding site has later been determined to ATTGCGCAAT (Agre et al. 1989). All C/EBP members have the same DNA-binding motif except for C/EBPζ that lack DNA binding possibilities. Two isoforms of C/EBPα are generated from the mRNA, one full-length protein of 42 kDa and a shorter form of 30 kDa. This shorter form has the same DNA-binding domain but an altered transactivation potential compared to the full-length C/EBPα (Lin et al. 1993, Ossipow et al. 1993). C/EBPα was first found to be expressed in cells that are involved in liver metabolism and were specifically able to regulate the expression of genes involved in these metabolic processes (McKnight et al. 1989). In fact, C/EBPα knockout mice usually die about eight hours after birth due to impaired expression of glycogen synthase, which leads to hypoglycemia (Wang et al. 1995). C/EBPα also plays an important role in liver-, adipose- and intestinal tissue differentiation (Samuelsson et al. 1991, Chandrasekaran et al. 1993, Flodby et al. 1996).

The C/EBPβ gene also codes for two protein isoforms. The full-length 38-34 kDa protein is called liver enriched activator protein (LAP) and a truncated 16-kDa form is called liver enriched inhibitory protein (LIP) (Descombes et al. 1991). The ratio of LAP-LIP hetero-dimerization complexes has been suggested to be important for the rate of transcriptional activation (Raught et al. 1995). C/EBPβ was first referred to as an acute phase response gene in cytokine-induced gene expression (Akira et al. 1990, Poli et al. 1990) and later as an activator of proliferation in pre-adipose cells (Cao et al. 1991, Umek et al. 1991). Moreover, after partial hepatectomy C/EBPα was downregulated, and C/EBPβ and δ were activated during the regeneration period (Mischoulon et al. 1992, Flodby et al. 1993). The role for C/EBPs during tumor development is largely unknown but preliminary studies on cell specific expression patterns of the different proteins suggest several possible functions for these family members. C/EBPζ has been reported to be present in myxoid liposarcoma (Aman et al. 1992) and Ewing’s sarcoma (Crozat et al. 1993). The shorter form of C/EBPβ, LIP, was detected in human breast tumors (Zahnow et al. 1997). In liver carcinomas, the expression of C/EBPα decreased in relation to tumor dedifferentiation (Xu et al. 1994). The anti-proliferating and anti-tumor properties of C/EBPα were further demonstrated by the induction
of C/EBPα in tumor cell lines (Timchenko et al. 1996, Watkins et al. 1996). This induction was correlated to increase of the cell cycle protein p21, which is also regulated by the tumor suppressor p53.

C/EBP IN THE OVARY

All of the C/EBP family members are expressed in the ovary. The C/EBPα protein expression increase during folliculogenesis and decline progressively after the LH-surge (Piontkewitz et al. 1993, Sirois et al. 1993). After ovulation, C/EBPα increase during the formation of the corpus luteum. C/EBPα is mainly located to GCs and TCs of the preovulatory follicle. It was further shown that administration of antisense-C/EBPα into the ovarian bursa, reduce ovulation by 50% (Piontkewitz et al. 1996a). Entrapped oocytes were found in large follicles and there was also fewer corpora lutea than in ovaries of control animals.

The expression of C/EBPβ mRNA and protein on the other hand was induced by human corion gonadotropin (hCG) in isolated rat GCs (Sirois et al. 1993). In intact animals, C/EBPβ expression was also shown to increase in response to hCG injection in rat and mouse GCs (Pall et al. 1997, Sterneck et al. 1997). The function of this might be tightly correlated to ovulation since both Sterneck et al (1997) and Pall et al (1997) could with different approaches show decreased ovulation rate when C/EBPβ expression was absent (C/EBPβ-/- mice) or impaired (antisense C/EBPβ ). These studies also demonstrated entrapped oocytes in the follicles and the C/EBPβ -/- mice lacked corpora lutea. A possible target gene for C/EBPβ in the ovulatory process is prostaglandin synthase-2 (PGS-2) (Sirois et al. 1993).

AIMS OF THE PRESENT STUDY

INTRODUCTION

During the ovarian events of follicular development, ovulation and corpus luteum formation, the cells of both the follicular and stromal compartments undergo extensive morphological changes. This is known to involve regulation of protein and gene expression responsible for proliferation and differentiation of the individual cells. Less is known in the field of cell-cell adhesion during these processes. The growing scientific work concerning roles of specific families of cell-cell adhesion molecules (CAM) address questions not only concerning pure mechanical cell-cell adhesion but also the role for CAMs in more complex cell processes such as differentiation, proliferation, invasion and apoptosis. In order to investigate the role for cadherins in the normal ovarian cycle, paper I describe the time- and cell-specific expression of the E-cadherin/catenin complex during folliculogenesis, ovulation and corpus luteum formation.

E-cadherin is known to be important for proper development of epithelium during embryogenesis, but it is also stated that downregulation of E-cadherin is a prerequisite for
mesenchymal development. Loss or blockage of E-cadherin expression in normal epithelial or
tumor cell-lines change these cells to a more fibroblast-like cell type with less cell-cell
adhesion and invasive capacities. This epithelial-mesenchymal conversion may also occur
during tumour progression. The benign adenomas and the highly differentiated
adenocarcinomas of the ovary, have a more restored epithelial morphology while the poorly
differentiated adenocarcinomas resemble mesenchymal tissue. In many other types of
epithelial-derived malignancies, E-cadherin is downregulated or lost during tumour progression
and this correlates to a changed phenotype. Paper II and III addresses the question of E-
cadherin expression in the normal human ovary and ovarian tumors of different grades and
stages.

The expression of most proteins in the cell is tightly regulated. In order to initiate
transcription of the targeted gene, a set of transcription factors and other controlling elements
have to bind to that specific gene’s promoter region on the DNA. This event is followed by
transcription of the gene, RNA processing of the primary transcript, transport of mRNA to the
cytosol, translation of the protein and protein activation/inactivation by mechanisms such as
phosphorylation. Each of these steps can be controlled by other sets of proteins.

Regulation of E-cadherin gene expression was, at the time for initiation of this thesis not very
well known. The intracellular binding proteins, the catenins, were suggested to control the
adhesive properties of the complex. Others suggested regulation on the level of protein
translation or DNA transcription. In fact, early promoter studies had shown binding consensus
sites for the myc and C/EBP families of transcription factors, both with specific relevance to
the ovary. The proto-oncogene protein c-Myc, which stimulates cell-proliferation, was shown
to be amplified and overexpressed in ovarian tumors. The different C/EBP family members
were suggested to be involved in differentiation and proliferation during normal follicular
development and ovulation. Paper IV describes the time and cell-specific expression of C-
myc during folliculogenesis and corpus luteum formation and paper V describes the
expression of members of the C/EBP family in the normal human ovary and ovarian tumors
of different grades and stages.

SUMMARY OF AIMS

- To investigate the time- and cell-specific expression of cell-cell adhesion factors in the
  rat ovary during folliculogenesis, ovulation and corpus luteum formation.

- To investigate the expression of cell-cell adhesion factors in the normal human ovary,
  in comparison to benign and malignant ovarian tumors of epithelial origin.

- To investigate the time- and cell-specific expression of the transcription factor C-myc
  in rat ovary during folliculogenesis, ovulation and corpus luteum formation.

- To investigate the expression of the C/EBP family of transcription factors in the
  normal human ovary, in comparison to benign and malignant ovarian tumors of
  epithelial origin.

METHODOLOGICAL CONSIDERATIONS
The methods used in this thesis are described in detail in the Material and Methods sections of the individual papers. Some of the aspects of these methods and techniques are discussed in a more general aspect below.

ANIMALS AND EXPERIMENTAL PROTOCOLS

The different models used to initiate follicular growth, ovulation and corpus luteum formation are well established and have previously been characterized in detail (Richards 1975, Nordenström 1981, Billig et al. 1993). The reason for using several models was two-fold. Firstly, it was important to assure that the results and findings from one model (the PMSG-model) of folliculogenesis and corpus luteum formation could be repeated with the second model (estradiol and FSH treated hypophysectomized rats) (paper IV). Secondly, the effect of estrogen on the expression of CAMs was of specific interest (paper I).

HUMAN TISSUES

Tissue samples were collected from the patients that were admitted to Sahlgrenska University Hospital for operations due to ovarian tumors (papers II, III and V). Normal ovarian tissue samples were taken from patients undergoing laparotomy for benign non ovarian disease (papers II and V). The tumors were classified as benign adenomas, borderline tumors and adenocarcinomas according to well-accepted criteria (Kjellgren and Angström 1995). Furthermore, the tumors were staged and graded according to the International Federation of Gynecology and Obstetrics (FIGO) classification.

TISSUE PREPARATION

The dissection procedures of granulosa (GC)-, theca (TC) cells and residual ovary (ROV), were performed according to well-established techniques (Piontkewitz et al. 1993). To minimize contamination of other types of cells in the different fractions, the dissection procedures were performed under microscope (Nordenström 1981). For each time-point, tissues and cells from 3-5 animals were pooled.

Ovarian tissues and cells were prepared for mRNA analysis by mechanical and chemical homogenization and RNA extraction with phenol-chloroform according to Chomcynski and Sacchi. Levels of mRNA were related to the concentration of total nucleic acid (TNA). Tissue extracts from both human and rat was prepared for protein analysis by mechanical homogenization of the tissue in a buffer protecting the proteins from degradation by proteinases. The cells in the homogenate, were further lysed both chemically and by sonication before protein concentration was evaluated (Piontkewitz et al. 1997).

SOLUTION HYBRIDIZATION ASSAY

This method is used to measure small quantities of mRNA. The mRNA is hybridized with a $^35$ labeled antisense RNA probe and the individual concentrations of each sample are related to a standard curve made from the probe’s sense RNA. This gives a reliable quantification of the mRNA. In this assay it is important to analyze the qualitative aspects of the labeled RNA (size of transcript) to ascertain probe specificity.

NORTHERN BLOT ANALYSIS
With this technique, equal amounts of RNA can be separated by electrophoresis, blotted/transferred to a membrane and detected by hybridization to a \(^{32}\text{P}\) labeled RNA or DNA probe. Hybridized bands are detected on X-ray films. This method provides information of the size of the transcript that can be detected with the specific probe and if there are any splice variants of different sizes present. In Northern blot analysis the amount of RNA needed is much larger than in solution hybridization assays and even though densitometric scanning enables you to compare in-between samples, the latter method is preferred in quantitative analysis.

**IMMUNOBLOTTING**

This method makes it possible to separate equal amounts of denatured proteins by electrophoresis and transfer them to a membrane. The protein is then detected with antibodies linked to alkaline phosphatase enzyme, which can emit chemiluminescence in the presence of certain chemicals. The bands are visualized on X-ray film. With immunoblotting, protein levels can be determined and semi-quantitatively measured by densitometric scanning. By adding a standard probe of different sizes, this method also provides information of protein size.

**ASSAY OF SOLUBLE E-CADHERIN**

The method of enzyme-linked immunosorbent assays (ELISA) provides a sensitive way to determine the concentration of proteins in liquid solutions. Even extremely low amounts of a protein can be detected by this technique.

**IMMUNOHISTOCHEMISTRY**

In this technique antigens in a tissue section is detected by the precise specificity of an antibody labeled with fluorescent dyes (FITC). With a fluorescence (UV-light) microscope evaluation of the cell-specific localization of a molecule can be achieved. The sensitivity of antibodies is enhanced by a signal-amplification method that provides binding of multiple secondary antibodies to the primary unlabeled antigen-antibody complex (i.e. biotin-streptavidin-FITC). This method can be used indirectly for quantification but is most widely used to specify the location of a protein.

**IN SITU DNA 3’-END LABELING**

When a cell enters apoptosis, programmed cell death, the DNA breaks apart into smaller fragments. This can be detected by an antibody directed to the 3’-end of the DNA fragments and visualized by colored dyes. With this sensitive method, it is possible to
determine which specific tissue and/or which specific cells that has entered the apoptotic pathway. You can also indirectly use this method as a quantitative assay.

RESULTS AND COMMENTS

In this section, the results of papers I-V are summarized and commented on. In addition some unpublished data of relevance will be presented.

THE CELL- AND TIME-SPECIFIC EXPRESSION AND LOCALIZATION OF E-CADHERIN, α - AND β -CATENIN IN THE RAT OVARY (PAPER I)

Cell adhesion junctions in the ovary, and in particular among the granulosa cells (GCs), have been carefully examined by the use of electron microscopy. The profound morphological and functional changes that ovarian cells undergo during folliculogenesis, ovulation and corpus luteum formation suggest a role for cell-cell adhesion molecules (CAMs) in these processes. Not much was known about the specific location or the hormonal regulation of E-cadherin and the catenins during the ovarian cycle, when this study was conducted. Ovaries were analyzed from gonadotropin- and estrogen-stimulated rats at different time-points of the ovarian cycle (for details see paper I).

By use of immunohistochemistry techniques, staining for E-cadherin were demonstrated in theca (TC)-, interstitial-cells and surface epithelium (OSE). E-cadherin was not detected in GCs except for GCs of small follicles located in the inner, cortical region of the ovary. This transient expression of E-cadherin in GCs could be of specific importance to the cortically located primordial and preantral follicles. The expression of E-cadherin protein was further analyzed with immunoblotting techniques but no stimulatory effect could be demonstrated after hormonal treatment. Apoptotic follicles undergoing atresia were also without E-cadherin.

All of the ovarian cell compartments stained positively for α - and β -catenin. The expression of α -catenin was affected by hormones, as demonstrated by a decrease in GCs after both human chorion gonadotropin (hCG) and diethylstilbestrol (DES) treatment while the expression of β -catenin was unaffected. Prior to ovulation, there was a less intense staining of both α - and β -catenin and to some extent also E-cadherin, which was restricted to the TCs. This interesting finding suggests that downregulation of cadherin/catenin complexes could facilitate the morphological changes that occur prior to ovulation.

Corpus luteum (CL) of ovaries from cycling rats was investigated by immunohistochemistry. Positive staining of E-cadherin, α - and β -catenin was demonstrated in all of the investigated CL. The intensity was, however, lower than in adjacent interstitial tissue or TCs of nearby follicles.

EXPRESSION OF E-CADHERIN IN THE HUMAN OVARY AND OVARIAN TUMORS (PAPER II AND III)
Even though the ovary is an organ of mostly mesoderm (mesenchymal) origin, the most common type of ovarian tumors is the epithelial type. These tumors are described as epithelial derived, since they morphologically resemble adenomas and adenocarcinomas and since they also express characteristic epithelial markers. The cell-cell adhesion molecule E-cadherin has been described as a marker for epithelial cells that decreases in tissue levels with the dedifferentiation from a highly to a poorly differentiated adenocarcinoma. In paper II, the expression and cell specific localization of E-cadherin in the normal human ovary and ovarian tumors of epithelial origin was investigated.

By the method of immunohistochemistry, it was demonstrated that the ovarian surface epithelium (OSE) in general is without E-cadherin staining. However, E-cadherin was up regulated and present in deep cleft formations of OSE and in inclusion cysts lined with OSE in the normal ovary. Preliminary results from primary cultures of normal human OSE (nOSE) and an immortalized OSE (iOSE) cell-line demonstrate cells without E-cadherin staining or expression (Ivarsson, Sundfeldt et al unpublished results). Stimulation by growth factors or cytokines such as insulin like growth factor 1 (IGF-1), interleukin-8 (IL-8), tumor necrosis factor $\alpha$ (TNF$\alpha$) and epidermal growth factor (EGF) did not induce E-cadherin in the iOSE cells (Ivarsson, Sundfeldt et al unpublished results).

The E-cadherin staining of the primary tumors of all grades and stages was epithelial-specific and strong except for in one undifferentiated adenocarcinoma, where the staining was more punctuated. Two peritoneal metastasis (stage III) of moderately/poorly differentiated adenocarcinoma were also analyzed and demonstrated areas of lesser E-cadherin staining as compared to the primary tumor. By immunoblotting, an increase of E-cadherin expression was demonstrated in the more malignant ovarian tumors as compared to the more benign tumors. There was also expression of E-cadherin in an ovarian cancer cell line (OVCAR3-NIH) but not in a stroma-derived thecoma tumor. With immunoblotting, the lower levels of E-cadherin in metastasis could also be demonstrated.

Since the ovarian tumors with acquired invasive capacity (stage III) and the poorly- and undifferentiated tumors still expressed E-cadherin, other components of this adhesion complex could be influenced. In a not yet completed study, we have looked at the expression and cellular localization of $\alpha$ - and $\beta$ -catenin in the normal ovary and ovarian tumors. Preliminary results demonstrate both $\alpha$ - and $\beta$ -catenin in OSE, deep cleft formations and in inclusion cysts of the normal ovary (Figure 4 a, b and 5 a, b). The expression of $\alpha$ -catenin is strong in the malignant tumors with a few exceptions, where it is either totally absent or lost in parts of the tumor (Figure 4 c, d, f and Figure 6 a-d). These particular tumor samples have been analyzed for mutational screening in collaboration with Dr. Nollet (University of Ghent, Belgium), with negative results (preliminary findings). Beta-catenin seems to be upregulated in the more malignant tumors. Recently, a role for $\beta$ -catenin in tumor progression was suggested. Due to mutations, the tumor cells i.e. colon cancer cells, have large free pools of cytoplasmic $\beta$ -catenin that can interact with the transcription factor Tcf-Lef. The $\beta$ -catenin/Tcf-Lef complex would then translocate to the cell nucleus and activate transcription.

By separating tumor tissue into three different fractions (cell membrane, cytosol and nucleus), we could by immunoblotting demonstrate increased levels of $\beta$ -catenin in the cytosol and nucleus of the malignant tumors (Figure 7) as compared to benign tumors and normal ovary (unpublished data).

In paper III, we further analyzed the concentration of soluble (s) E-cadherin in peripheral blood, cystic-, and ascitic-fluids from patients with benign, borderline and
malignant cystic ovarian tumors. In light of the results of paper II, was an increase of sE-cadherin in all fluids from the more malignant tumors, expected. Concentrations of sE-cadherin in the fluids were measured by enzyme-linked immunosorbent assay (ELISA). Soluble E-cadherin was detected in all samples analyzed. The concentration of sE-cadherin was higher in cystic fluid from borderline tumors and adenocarcinomas when compared to benign cystadenomas. No differences in the levels of sE-cadherin were seen between the tumor groups regarding levels in ascites or peripheral blood. However, the ratios of cystic fluid/peripheral blood levels were also higher in borderline tumors and adenocarcinomas compared to benign cystadenomas. The results of this study suggest a possibility for sE-cadherin to be beneficial in increasing the accuracy during preoperative diagnosis of adnexal masses.

**THE CELL- AND TIME-SPECIFIC EXPRESSION AND LOCALIZATION OF THE PROTO-ONCOGENE C-MYC IN THE OVARY (PAPER IV)**

The proto-oncogene protein c-Myc is a transcription factor important for proliferation of cells, as it is believed to bring cells through the G1 checkpoint, which subsequently leads to initiation of mitosis. Both follicle and corpus luteum development involve proliferation of the cells, in particular the GCs of the follicle and endothelial cells of the corpus luteum. In paper IV, we could demonstrate an increase of both c-Myc mRNA and protein during the formation of the preovulatory follicle. These findings were based on two different rat models of induced folliculogenesis in immature prepubertal rats (for details see paper IV). By separation of the follicle cells into GCs and residual ovary (ROV), we could further show that the c-Myc increase was localized to the GCs at both mRNA and protein levels.

After the injection of hCG, to mimic the LH-surge, c-Myc mRNA and protein levels were rapidly induced in both GCs and ROV suggesting an increased proliferation of the cells during the very initial steps of corpus luteum formation. The proliferative status of the cells was further demonstrated by the expression of proliferating cell nuclear antigen (PCNA) by immunoblotting which increased at the same time-points as c-Myc. PCNA has been shown to be a marker of cells that are in S-phase of the cell cycle. The levels of c-Myc returned to the preovulatory level just prior to ovulation. During the first days of pseudopregnancy c-Myc expression resumed its high levels. Figure 8 gives a simplified view of the changes in c-Myc expression during folliculogenesis, ovulation and corpus luteum formation, which are presented in paper IV.
Figure 8. C-Myc mRNA and protein in expression in the rat ovary.

EXPRESSION OF CCAAT/ENHANCER BINDING PROTEIN (C/EBP) FAMILY OF TRANSCRIPTION FACTORS IN THE HUMAN OVARY AND OVARIAN TUMORS (PAPER V)

The knowledge about the C/EBP family of transcription factors has rapidly increased since C/EBP was first discovered in 1988. Their roles in proliferation and differentiation of normal ovarian cells have been well documented but possible functions during tumorigenesis are largely unknown. Since C/EBPs take part on the transcriptional level in key processes concerning the cell cycle they can be regarded as potential oncogenes.

In paper V, tissue samples from normal human ovaries, benign-, borderline- and malignant ovarian tumors of different grades and stages were investigated for protein expression and cell specific staining of the C/EBP family of transcription factors (α , β , δ and ζ ). By immunohistochemistry, we could localize the C/EBPs mainly to the normal epithelium and epithelial-derived tumor cells even though C/EBPδ and ζ to a lesser extent also were found in the surrounding stromal tissue. The intracellular localization of C/EBPα was in the cytosol of the cells, in contrast to C/EBPβ and C/EBPδ, which stained the nucleus of all samples. C/EBPζ was predominantly localized in a perinuclear fashion in both stromal and epithelial cells.

Semi-quantitative measurements of the protein levels with immunoblot technique could also demonstrate expression of the C/EBPs in a majority of the tissue samples. The amount of C/EBPβ increased during epithelial tumor progression, while C/EBPα and δ levels were more
or less constant. A slight increase in the amount of C/EBPζ was also detected in the more malignant samples of ovarian tumors. These results suggest a role for C/EBPβ during the proliferative process of epithelial-derived ovarian tumors and a role for the C/EBPβ levels as a potential malignant marker for these tumor cells. The fact that i.e. C/EBPα levels were constant and C/EBPζ levels even slightly increased, propose functions for these proteins as negative regulators in tumorigenesis.

Figure 4. Staining of α -catenin in human ovarian tumors and in normal ovaries by immunohistochemical analysis of, a, normal ovary with OSE; b, ovary with inclusioncysts lined with OSE; c, borderline type tumor, stage I; d, peritoneal metastasis from a moderately/poorly differentiated adenocarcinoma; e, moderately/poorly differentiated adenocarcinoma; stage III; f, undifferentiated adenocarcinoma, stage III.

Figure 5. Staining of β -catenin in human ovarian tumors and in normal ovaries by immunohistochemical analysis of, a, normal ovary with OSE; b, ovary with inclusioncysts lined with OSE; c, borderline type tumor, stage I; d, peritoneal metastasis from a moderately/poorly differentiated adenocarcinoma; e, moderately/poorly differentiated adenocarcinoma; stage III; f, undifferentiated adenocarcinoma, stage III.

Figure 6. Immunohistochemical staining of a moderately differentiated adenocarcinoma, stage III with, a; cytokeratin (pos. control); b, E-cadherin (positive); c, α -catenin (negative); d, β -catenin (both positive and negative areas).

Figure 7. Immunoblot expression of β -catenin in sub-cellular fractions of a normal ovary and different ovarian tumors as indicated.
GENERAL DISCUSSION

CELL ADHESION MOLECULES (CAMs) IN FOLLICULOGENESIS, OVULATION AND CORPUS
LUTEUM FORMATION

In paper I it was found that E-cadherin was not expressed in granulosa cells (GCs) of growing antral follicles. Granulosa cells proliferate and differentiate with the development of the follicle from the antral to the preovulatory stage. Cadherins were earlier described to increase (Blaschuk et al. 1989, Farookhi and Blaschuk 1989) in cultured GCs in vitro stimulated in the presence of estradiol or diethylstilbestrol (DES). In one study, anti-sera against avian N-cadherin, recognizing several cadherins, was used (Blaschuk et al. 1989) so the specific cadherin expression could not be evaluated. However, N-cadherin was later described in GCs (Farookhi and Blaschuk 1991, Peluso et al. 1996, Farookhi et al. 1997, Trolle et al. 1997) and was regulated by estradiol (MacCalman et al. 1995). It is possible that the immunoreactivity detected was mostly due to the N-cadherin CAM. The other study (Farookhi and Blaschuk 1989) used an antibody specific for E-cadherin, but it should be noted that expression of E-cadherin was only found in GCs that were aggregated in culture and not in disaggregated cells. This observation does of course not reflect the in vivo situation, where the GCs will become more and more loosely packed in the growing follicle (Bjersing et al. 1974a, 1974b). Moreover, adherens junctions that are build up by E-cadherin are downregulated with follicular growth (Albertini et al. 1974, Amsterdam et al. 1992) at the same time as there are a growing number of gap junctions (Amsterdam et al. 1987, Grazul Bilska et al. 1997). Interestingly, N-cadherin is more often, compared E-cadherin, situated at non-junctional sites (Geiger et al. 1992, Salomon et al. 1992) and this could justify its presence in GCs. In a preliminary report (Machell et al. 1997), the same group who previously described E-cadherin in GCs actually, by immunohistochemistry, describes the absence of E-cadherin in GCs but presence of N- and K-cadherin. Moreover, they state later that N-cadherin is the only cadherin present in GCs (Harandian et al. 1998).

There might be species differences though, since E-cadherin was described in GCs of porcine ovaries (Ryan et al. 1996). The species differences are further noted by the expression E-cadherin in ovarian surface epithelium (OSE) of rat (Hoffman et al. 1993) paper I), mouse (Damjanov et al. 1986) and pig (Ryan et al. 1996), while normal human OSE was without E-cadherin expression (Auersperg et al. 1994, Maines-Bandiera et al. 1997, Davies et al. 1998, Wong et al. 1999)(paper II). In contrast there was no difference in expression of E-cadherin theca cells (TCs) or interstitial cells between porcine and rat ovaries (Ryan et al. 1996) paper I) and the localization to corpus luteum was similar in primates and rats (Khan-Dawood et al. 1996a, 1996b) paper I). Thus, the cadherin group of proteins seems to be present in all cell-types of the ovary, though the expression patterns change within the different cell-types. However, the simultaneous expression of α - and β -catenin (Butz et al. 1995) and paper I) support a functional role of the cadherins, in the ovary. The catenins bind to the cytoplasmic tail of cadherins and are necessary for the adhesive function (Ozawa et al. 1989, Gumbiner et
al. 1993) for both E-cadherin and N-cadherin (Wheelock et al. 1991). The cadherin/catenin complex bind to the cytoskeleton via actin (Rimm et al. 1995a) and this has been suggested to be vital for the strength and the function of cell-cell adhesion (Yap 1998). The expression of catenins in GCs, as described in paper I, supports a role in conjunction with cadherins in these cells.

In the in vivo study on normal follicular development in the rat (paper I), E-cadherin staining in epithelial cells/GCs of small follicles situated in the cortex of the ovary was demonstrated. It was also noted that E-cadherin disappeared in GCs of antral follicles. In line with this, fetal porcine ovaries had much higher levels of E-cadherin than ovaries from fertile pigs (Ryan et al. 1996). However, both E- and N-cadherin mRNA levels in whole ovaries from immature mice was found to increase as a result of estradiol injection (MacCalman et al. 1994, 1995). The change of E-cadherin expression in primordial to antral follicles as described in paper I, could reflect a possible vital function for this CAM during early stages of follicular development. Firstly, cells of small primordial follicles are at a resting stage while GCs proliferate rapidly during follicle development (Richards 1980). Several studies have described increased cell proliferation after blocked or perturbed expression of cadherin in both normal (Hermiston et al. 1995a, 1995b) and tumor cells (Birchmeier et al. 1994a, Mareel et al. 1995, Perl et al. 1998) (discussed in detail later). Secondly, both N- and E-cadherin may protect intestinal cells and GCs from entering programmed cell death (Peluso et al. 1994, 1996, Hermiston et al. 1995a, Peluso 1997), suggesting a survival-promoting role for the cadherins in these particular cells. Thirdly, the disappearance of E-cadherin could be just a reflection of the morphological changes of GCs in the growing follicle.

The role for cell-adhesion in GCs during folliculogenesis was investigated by examining the intermediate filaments, i.e. actin, which was downregulated after gonadotropin stimulation of rat GCs in vitro with a simultaneous increase in steroidogenesis (Ben Ze'ev et al. 1987). These results is supported by the findings of paper I, where it is clearly demonstrated that α-catenin decreased in GCs after human chorion gonadotropin (hCG) or DES treatment. The influence of these hormones was specific for α-catenin since the expression of β-catenin was unchanged. This strengthens the hypothesis that less adherens junction mediated adhesion is necessary for GCs of the growing follicle.

The expression of E-cadherin/catenin complex in the TCs could be mostly of mechanical importance (Adams et al. 1998) to maintain structural integrity of the growing follicle. The complex could further help support the "blood-follicle barrier" (Powers et al. 1996) between GCs and TC interna cell layers, and protect GCs from influences of the immune system until ovulation (Espey 1980). The downregulation of E-cadherin/catenin complex in TC, only hours before ovulation, as was found in paper I could most likely facilitate the influx of immune cells, which promote the ovulatory process (Bramstrom et al. 1993). Morphological changes that relate to the development of the corpora lutea might also be affected (Jablonka Shariff et al. 1993). The E-cadherin/catenin complex was demonstrated in corpus luteum of rat ovaries (paper II) and primates (Khan-Dawood et al. 1996a, 1996b). However the staining was less intense when compared to adjacent interstitial cells and TCs of nearby follicles (paper II).

NORMAL OVARIAN SURFACE EPITHELIUM (OSE): THE CELLULAR ORIGIN FOR NEOPLASTIC INITIATION AND PROGRESSION?

During fetal development the coelomic epithelium, which gives rise to the OSE, has the capacity to differentiate into many different cell types. This capacity probably contributes to the large variety of phenotypes found among OSE-derived ovarian carcinomas (Nicosia et al. 1991, Blaustein 1995, Auersperg et al. 1995b). The adult OSE is also referred to as a germlinal epithelium since it still shares characteristics with the mesothel lining the peritoneum and normal epithelium (Blaustein et al. 1979, Nicosia et al. 1991). However, in the course of carcinogenesis, OSE become more committed to an epithelial phenotype than to undergo epithelial-mesenchymal conversion. Transformed OSE, and particularly ovarian...
carcinoma cells, maintain some epithelial characteristics in culture longer than normal OSE (Auersperg et al. 1994). The epithelial characteristics of ovarian carcinomas tends to be not only more stable but also more highly differentiated than in OSE, since more epithelial markers are expressed and a more complex epithelial morphology is seen (van Niekerk et al. 1993, Dyck et al. 1996, Auersperg et al. 1996). This characteristic distinguishes ovarian carcinomas from most other cancers, which in the course of neoplastic progression, become less differentiated than the epithelium from which they arise. In this aspect, the finding that E-cadherin is absent in OSE (Shimoyama et al. 1989, Auersperg et al. 1994) paper II) is not surprising. Mesothelial cells, mesothelioma cell lines and pleural mesotheliomas do not contain E-cadherin, but express N-cadherin (Pelin et al. 1994, Peralta Soler et al. 1995, Schofield et al. 1997). N-cadherin has also been detected in human OSE (Trolice et al. 1997, Wong et al. 1999).

The appearance of E-cadherin in cuboidal or tall cells, cleft formations and inclusion cysts of the normal ovary is quite intriguing (Maines-Bandiera et al. 1997, Davies et al. 1998) and paper II. Just recently, E-cadherin was detected more frequent in cultured OSE from patients with a family history of ovarian cancer (FHO) as compared to OSE from control patients (Wong et al. 1999). Mutations in the breast related carcinoma gene-1 (BRCA1) were found in 10/14 of patients with FHO. Whether this induction of E-cadherin expression is functionally significant in the neoplastic transformation of the ovarian epithelium or merely contributes to or results from the process of metaplasia of ovarian epithelium is, however, not known. Actually, E-cadherin has been suggested to be a "master-gene", which can induce an epithelial phenotype when upregulated (Birchmeier et al. 1991, Vandenburg et al. 1996). This mesenchymal/epithelial conversion of cells caused by E-cadherin have been studied by cDNA transfection of fibroblastic cell-lines, i.e. L-cells or 3T3-cells (Nagafuchi et al. 1987, Nose et al. 1988) and metastatic tumor cell-lines (Fri xen et al. 1991, Vleminckx et al. 1991, Takeichi 1993, Birchmeier et al. 1994b). These studies described a number of epithelial characteristics, like assembly into epitheloid aggregates, apical basal polarity, appearance of junctions and desmosomes and decreased invasiveness in tumor cell-lines, as a result of E-cadherin expression or re-expression. Tissue-specific overexpression of E-cadherin in mouse intestinal epithelium also results in slower cell migration (Hermiston et al. 1996). On the other hand, when a dominant negative mutant N-cadherin that block E-cadherin expression was used, epithelial morphology was changed and migration increased (Hermiston et al. 1995a).

Based on histopathological studies, early malignant changes characteristically seem to occur in OSE-lined clefts and inclusion cysts (Radisavljevic 1976, Scully 1995) and this sometimes occur in direct continuation from OSE to fully malignant tumors (Puls et al. 1992, Godwin et al. 1993). This suggests that upregulation of E-cadherin in normal OSE surrounded by stromal cells, could play a role in early events leading to the malignant phenotype. Genetic changes in normal cells, equivalent to the changes in the adjacent tumor, have been described in breast carcinomas (Deng et al. 1996). When a dominant negative E-cadherin mutant was introduced to keratinocytes, cell adhesion was lost, proliferation was inhibited, and terminal differentiation was stimulated (Zhu et al. 1996). However, according to the few reports on E-cadherins role in tumor initiation (Hermiston et al. 1995b, Guilford et al. 1998) or progression (Perl et al. 1998, Tlsty 1998) presented so far, a loss of E-cadherin rather than an upregulation would be the cause for tumor development.

The absence of E-cadherin in OSE, likely reflects the immature nature of human OSE and/or that these cells represent two different cell-types. One cuboidal/tall resting cell with CAM expression and one flat squamous cell that associate with the site of ovulation (Gillett et al. 1991). The downregulation of E-cadherin has also been described in wound healing of gastric ulcers (Hanby et al. 1994). Moreover, stimulation of growth factors which are produced either by the underlying stroma (Kruk et al. 1994, Vigne et al. 1994, Karlan et al. 1995a) or the OSE itself (Kruk et al. 1992, Lidor et al. 1993, Ziltener et al. 1993, Auersperg et al. 1995a) could promote E-cadherin expression. In particular EGF, EGF plus IGF-I (Berkuch et al. 1993, Mareel et al. 1994) and transforming growth factors (TGFα, TGFβ) (Jindal et al. 1994) increased proliferation while TGFβ (Berkuch et al. 1992) inhibited and steroid hormones (estrogen and progesterone) (Karlan et al. 1995b) had no effect on proliferation of OSE in culture. The possible accumulation of growth factors and hormones in the inclusion/cyst fluid could have a tumor promoting effect, especially if the genome already has acquired neoplastic mutations.

E-CADHERIN EXPRESSION DURING TUMOR PROGRESSION
The expression of E-cadherin was found in epithelial-derived benign adenomas and borderline tumors of the ovary (paper II, (Darai et al. 1997, Peraltu Solet et al. 1997, Davies et al. 1998). Protein and mRNA expression was further induced in well-, moderately- and poorly- differentiated adenocarcinomas of the ovary (Inoue et al. 1991, Veatch et al. 1994, Fujimoto et al. 1997a) and paper II, while Davies et al (1998) in their material found that all poorly differentiated tumors were without E-cadherin. The extracellular part that can be proteolytically cleaved to a soluble form (Damsky et al. 1983) of E-cadherin (sE-cadherin) was found in peripheral blood, cystic and ascites fluid from patients with ovarian cystic tumor masses (Darai et al. 1997b, Fujimoto et al. 1997b) and paper II. Concentration of sE-cadherin was increased in cystic fluid from borderline tumors and adenocarcinomas when compared to benign adenomas but no changes were seen in ascites (paper III) or peripheral blood (Darai et al. 1998) and paper III. In a genetic screening of 63 carcinomas of the ovary, only one mutation was detected in the E-cadherin gene (Risinger et al. 1994). However, decrease of E-cadherin was described in peritoneal metastasis and ascites cells when compared to the primary tumor (Veatch et al. 1994, Fujimoto et al. 1997b) and paper II.

In contrast numerous reports have been published describing a decrease of E-cadherin in adenocarcinomas, which correlated to dedifferentiation and invasive capacity of the cancers of different organs such as bladder and prostate (Umbas et al. 1992, Pizarro et al. 1994, Rimm et al. 1995), colon (Dorudi et al. 1994, Kawanashi et al. 1991), extracellular domain (Kintner 1992) or point mutations in the Ca\(^{2+}\) binding domain all result in non functional adhesion (Birchmeier et al. 1994a, Berx et al. 1998). However, mutations and deletions in genes coding for the cadherin/catenin complex seem to be an exception rather than a rule in cancer cells in vivo. Screening of the E-cadherin gene has detected mutations only in a few cases (Berx et al. 1998) such as in some gastric cancers (Becker et al. 1994) and gastric cancer cell-lines (Oda et al. 1994), lobular breast cancer (Berx et al. 1995a), and endometrial cancer (Risinger et al. 1994). In the majority of tumors where E-cadherin expression was lost or distorted, mutations in the corresponding gene have not been found. In paper II, molecular weight of E-cadherin, analyzed by immunoblotting, did not differ between E-cadherin in normal ovary, benign or malignant tumors. This suggests that at least in our material any occurrence of large rearrangements of the gene was not present. This is in line with a previous study where only one mutation was detected in 63 investigated ovarian carcinomas (Risinger et al. 1994).

Mutations and deletions of either of the catenins have been described to disrupt interactions in the cadherin/catenin complex (Gumbiner et al. 1993, Huber et al. 1996a) and to alter adhesion in several cancer cell lines of gastric (Oyama et al. 1994, Kawanashi et al. 1995), breast (Bullions et al. 1997, Tsukatani et al. 1997) or colon origin (Breen et al. 1993, Vermeulen et al. 1995). Interestingly, E-cadherin expression remained strong and located to the cell membrane even though cell-cell adhesion was diminished or lost, but in some cases E-cadherin and either catenin were simultaneously downregulated (Shimoyama et al. 1992, Morton et al. 1993, Umbas et al. 1997). For endometrioid (3/6) but not serous (0/5) adenocarcinomas of the ovary, mutations have been found in the \(\beta\)-catenin gene (Palacios et al. 1998). The mutational screening of ovarian carcinomas with

It is also clear, however, that loss of differentiation of epithelial (carcinoma) cells can occur while E-cadherin expression is retained (Birchmeier et al. 1993, Shiozaki et al. 1996). The absence of downregulation during tumor progression like in ovarian carcinoma has also been described in studies of lung cancer (Shimoyama et al. 1989), colon cancer (Dorudi et al. 1993, Kinsella et al. 1993) and gastric cancer (Oka et al. 1992). However, in these studies E-cadherin was analyzed by immunological techniques. This does not exclude an altered function of E-cadherin (see discussion below).

**REGULATION OF E-CADHERIN EXPRESSION**

Aberrations that result in junctional defects and impaired cell-cell adhesion have been described at many cell-biological levels.

**Expression of E-cadherin in vitro without the cytoplasmic tail** (Nagafuchi et al. 1988, Ozawa et al. 1990), extracellular domain (Kintner 1992) or point mutations in the Ca\(^{2+}\) binding domain all result in non functional adhesion (Birchmeier et al. 1994a, Berx et al. 1998). However, mutations and deletions in genes coding for the cadherin/catenin complex seem to be an exception rather than a rule in cancer cells in vivo. Screening of the E-cadherin gene has detected mutations only in a few cases (Berx et al. 1998) such as in some gastric cancers (Becker et al. 1994) and gastric cancer cell-lines (Oda et al. 1994), lobular breast cancer (Berx et al. 1995a), and endometrial cancer (Risinger et al. 1994). In the majority of tumors where E-cadherin expression was lost or distorted, mutations in the corresponding gene have not been found. In paper II, molecular weight of E-cadherin, analyzed by immunoblotting, did not differ between E-cadherin in normal ovary, benign or malignant tumors. This suggests that at least in our material any occurrence of large rearrangements of the gene was not present. This is in line with a previous study where only one mutation was detected in 63 investigated ovarian carcinomas (Risinger et al. 1994).
demonstrated an inhibitory influence on cell-cell adhesion by tyrosine phosphorylation of E-cadherin. The first E-cadherin/catenin complexes are also substrates for kinases (Daniel et al. 1997). Furthermore, the α- and β-catenin protein was lost in 18% and 21% respectively in ovarian cancers (Davies et al. 1998). We have also found a loss of α- and/or β-catenin in some ovarian carcinomas (unpublished results), that was not related to stage or grade. However, in some tumors the expression of β-catenin was increased. These findings might indicate an altered function of the cadherin/catenin complex albeit the presence of E-cadherin.

Transcriptional regulation of the E-cadherin gene was also reported. The E-cadherin promoter has been cloned in the mouse (Behrens et al. 1991, Ringwald et al. 1991) and the human (Bussemaker et al. 1994, Berx et al. 1995b, Ji et al. 1997). Promoter assays of several cell lines confirmed that decreased mRNA expression was due to alterations of E-cadherin transcriptional regulation (Bussemaker et al. 1994, Hennig et al. 1995, Ji et al. 1997). The promoter of the murine E-cadherin had a GC-rich region, a CCAAT-box, an E-pal site, containing two E-box consensus binding sites and two AP-2 sites. The two latter regions were not fully conserved in the human sequence. (Hennig et al. 1996) Hennig et al (1996) proposed that transcription factors from the helix-loop-helix family i.e. the myc family could bind to the E-pal site, members of the C/EBP family could bind the CCAAT-box while the AP-2 protein could bind the AP-2 sites. However, the optimal binding site for C/EBP has later been determined to ATT GCG CAA T (Agre et al. 1989). In preliminary studies of the E-cadherin promoter by electrophoretic-mobility-shift assay (EMSA), we found binding to both the CCAAT-box and to a closely (87%) related sequence (CTT GCG GAA G) to the optimal C/EBP site, with nuclear extracts from an ovarian cancer cell-line (NIH-OVCAR3) (Rask, Sundfeldt, unpublished results). However, only the almost optimal C/EBP site in the E-cadherin promoter and not the CCAAT-box could super-shift with C/EBPα and C/EBPβ specific anti-serum (Rask, Sundfeldt, unpublished results). Recently, it was also shown that c-Myc, AP2 and the retinoblastoma gene product (RB) could activate transcription of the E-cadherin gene (Batsché et al. 1998). Moreover as Tef-Lef/β-catenin was found to bind the E-cadherin promoter an autocrine-regulatory-loop was suggested (Huber et al. 1996b). Whether transcriptional regulation of E-cadherin is altered in ovarian tumors remains to be elucidated.

Alternatively, other mechanisms may have overcome the invasive suppressor function of E-cadherin. Lateral clustering has, in cell culture experiments, demonstrated to influence cell adhesion strength without changes in expression of E-cadherin (Adams et al. 1998, Yap 1998). This occurs during compaction of blastomeres and can also be seen in cell culture systems (Adams et al. 1998). The significance of clustering and lateral dimerization in tumor progression has not been investigated but it could impair the adhesive function in dedifferentiated tumors, such as ovarian carcinomas, without changes in E-cadherin expression. The crystallographic structure of the extracellular cadherin has recently been elucidated and showed that cadherins bind in dimers to the cadherin dimers of the neighboring cell (Shapiro et al. 1995). This involves the specific histidine-alanine-vaneline (HAV) sequence (Büsschak et al. 1990, Shapiro et al. 1995) and synthetic decapeptides containing HAV inhibited E-cadherin mediated activities (Büsschak et al. 1990). This finding suggests that the extracellular proteolytically cleaved sE-cadherin also might have the ability to influence E-cadherin functions through its HAV sequence. The functional role of the 80 kDa form is not clear (Damsky et al. 1983, Takeichi 1988). It was demonstrated to have biological activities, since sE-cadherin was able to disrupt cell-cell adhesion in epithelial cell cultures (Wheelock et al. 1987). The increased concentration of sE-cadherin in blister fluids from patients with various cutaneous diseases further supported this theory (Matsuyoshi et al. 1995). The potential thus exists for a high turnover of E-cadherin in ovarian adenocarcinomas, with an increased proteolytic cleaving or shedding of sE-cadherin to the cystic fluid (paper III). This could in turn lead to a more invasive phenotype. The 80 kDa product has also been proposed to recruit more E-cadherin to the cell membrane (Näthke et al. 1993), which in ovarian tumor cells could turn out to induce a vicious circle.

E-cadherin/catenin complexes are also substrates for kinases (Daniel et al. 1997). Several groups have demonstrated an inhibitory influence on cell-cell adhesion by tyrosine phosphorylation of E-cadherin. The first experiments, where cellular tyrosine phosphorylation was increased, reduced cadherin mediated adhesive strength (Matsuyoshi et al. 1992, Hamaguchi et al. 1993, Behrens et al. 1993) but did not effect the level of E-cadherin expression. When tyrosine phosphorylation was blocked, cell adhesion was restored. Observations have suggested that regulation by phosphorylation were effectuated through β-catenin (Shibamoto et al. 1994, Daniel et al. 1997, Yap 1998) but also via p120cas (Shibamoto et al. 1995) that binds directly to the cytoplasmic
tail of E-cadherin (Reynolds et al. 1994, Daniel et al. 1995). A thorough review about cadherin/catenin regulation, phosphorylation via growth factors and cell signaling was presented by Daniels et al (1997). Recent findings of β -catenins role in the Wnt signaling pathway suggests modifications of this protein by other intracellular partners, such as glycogen synthase kinase 3β (GSK3β ) (BenZeev et al. 1998) affecting the amount of free β -catenin in the cytosol. In fact, Wnt signaling increased the free pool of β -catenin by dephosphorylation, which then could bind to the transcription factor T-cell factor-Leukocyte enhancer factor (Tcf-Lef) (BenZeev et al. 1998). The β -catenin/Tcf-Lef complex translocated to the nucleus and could regulate transcription of E-cadherin (Huber et al. 1996b), c-Myc (He et al. 1998) and cyclin D1 (Tetsu et al. 1999), the only targeted genes found so far. In our immunohistochemical analysis of ovarian carcinomas, β -catenin were observed mainly in the plasma membrane and not in the cytosol or nucleus (Figure 5). However, the ovarian cancer cell line (NIH-OVCAR3) and immunoblotting analysis of ovarian carcinoma cell fractions demonstrated β -catenin expression in all cell compartments (Figure 7) which then could be a result of changes in the Wnt signaling pathway.

The strong expression of E-cadherin in ovarian carcinomas could be a reflection of the earlier change from the mesenchymal OSE to the epithelial carcinoma and the fact that ovarian carcinomas retain many epithelial characteristics (see discussion about OSE) even at low differentiated grades. It could also be speculated that the ovarian carcinomas might benefit from expressing CAM during progress until the point or the location where invasion starts. A transient up- and downregulation of E-cadherin could actually promote proliferation of tumor cells and allow repeated steps of invasion (Giroldi et al. 1994-95, Mareel et al. 1995). As discussed in detail above, many findings suggest that functional adhesion may be impaired even when E-cadherin is expressed. If functional adhesion is impaired during ovarian carcinogenesis remains to be elucidated.

E-CADHERIN IN CLINICAL PRACTICE

E-cadherin as a prognostic marker for ovarian carcinomas seem less plausible since there was no correlation between expression of the protein or mRNA with dedifferentiation or invasive capacity (paper II) (Fujimoto et al. 1997a, Davies et al. 1998). The tumor-promoting role of E-cadherin-increase in OSE from patients with BRCA1 mutations (Wong et al. 1999) and expression in inclusioncysts of the normal ovary remains to be elucidated. It would be of great interest to find out whether patients with E-cadherin expression are prone to develop ovarian cancers or if it is just a reflection of a mesenchymal/epithelial conversion?

In paper III, the levels of the soluble part of E-cadherin (sE-cadherin) in peripheral blood, cystic and ascites fluid from patients with benign, borderline and malignant ovarian cystic tumors were investigated. The sE-cadherin is proteolytically cleaved and this cleavage was hypothesized to increase during tumor progression. This was demonstrated to be true in plasma from patients with a number of different cancers (Katayama et al. 1994) but not from patients with gastric or ovarian carcinoma (Velikova et al. 1997, Darai et al. 1998). Increased concentrations of sE-cadherin were also described in urine from patients with bladder cancer and in blister fluids from patients with cutaneous diseases (Banks et al. 1995, Matsuyoshi et al. 1995). Cystic fluid from ovarian tumors, concentrations of sE-cadherin were increased in borderline tumors and adenocarcinomas as compared to benign adenomas (Darai et al. 1998)(paper III). In paper III, no differences in concentrations between the patient groups were seen in peripheral blood or ascites fluid. The finding could however be useful in attempts to find a diagnostic tool, which can differentiate between benign cystic masses and borderline/malignant cystic masses before surgical intervention is decided upon (Chapron et al. 1996). Thus low concentrations of sE-cadherin would predict a benign cystadenoma rather than a borderline or malignant tumor. Cystic fluids from ovarian tumors have been found to contain several factors i.e. interleukin-8 (IL-8) (Ivarsson et al. 1998), IL-6 (van der Zee et al. 1995), and insulin-like growth factor-I (IGF-I) (Karaski et al. 1994) suggested as possible markers of ovarian tumor progression. However, a prospective study with a larger material of cystic fluids would be required before adapting these "markers" to clinical practice. In addition cyst fluids from simple cysts should also be included in such study.

THE PROTO ONCOGENE C-MYC AND FOLLICLE CELL PROLIFERATION
In the primordial follicle the single layer of GCs is quiescent (Hirshfield 1991). After a couple of slowly dividing cell cycles, GCs start to proliferate rapidly as a response to follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Richards 1980, Richards et al. 1995). Proliferation of GCs in rat during this phase is demonstrated by the concomitant increase in $^3$H-thymidine (Rao et al. 1978), 5-bromodeoxyuridine (BrdU) (Gaytan et al. 1996) or proliferating nuclear cell antigen (PCNA) (Chapman et al. 1994)(paper IV). This latter protein is involved in DNA synthesis and repair (Shivji et al. 1992). PCNA is commonly used as a marker of proliferation. In human ovaries proliferation in follicles was also shown by increased labeling indexes of Ki67, PCNA and silver staining of nuclear organizer regions (AgNORs) (Funayama et al. 1996).

Proliferation of GCs during follicular development were further demonstrated in cell cycle cyclin D2 (-/-) mice, were GC was unable to respond with normal proliferation after FSH stimulation (Sicinski et al. 1996). Moreover, cyclin D2 (Robker et al. 1998b) and cyclin E (Robker et al. 1998a) were hormonally induced during follicular growth and localized to proliferating granulosa cells. These two cyclins are positive regulators of cell cycle progression during the G1-phase (Sherr 1994).

In paper IV, we demonstrated that c-Myc, which is low in quiescent cells and increase rapidly after mitotic stimuli (Bouchard et al. 1998), is transiently increased in GCs after PMSG stimulation in the immature rat. By immunohistochemistry this increase was also primarily detected in the GCs. This response to PMSG had been demonstrated earlier for intact animals and for ovaries in peri-fusion (Delidow et al. 1990, 1992). In paper IV, there was a transient increase of c-Myc after PMSG. However according to Hirshfield et al (reviewed 1991)) the GCs in the antral follicles from the small immature ovary, belong to the 8th or 9th generation. This means that the proliferative potential of GCs in the PMSG-model is limited, since the preovulatory follicle contains GCs of the 10th generation (Hirschfield 1991). This could explain the transient and limited increase of c-Myc after PMSG in paper IV. Moreover, we could demonstrate an induction of c-Myc in ovaries from immature rats, 25 days of age, as compared to c-Myc levels in ovaries from immature rats, 18 days of age. Both c-Myc mRNA and protein were present in all follicles but primordial follicles in human ovaries (Putowski et al. 1997) and in GC and corpus luteum of primates (Fraser et al. 1995). C-Myc was not present in corpus albicans or in ovaries from postmenopausal women (Putowski et al. 1997). This strengthen the hypothesis that c-Myc is important during the proliferative stages of ovarian cell cycle but also that c-Myc might play a role in apoptosis since atretic follicles and corpus luteum expressed this proto-oncogene (Fraser et al. 1995, Putowski et al. 1997, Thompson 1998)

The GCs of the developing antral follicle not only proliferate but also differentiate into steroid producing cells and acquire LH-receptors (Uilenbroek et al. 1979, Richards 1980). The LH-surge reprograms the steroidogenic cells to produce mainly progesterone and to differentiate by luteinization. Both TCs, GCs and endothelial cells join in the formation of the mature corpus luteum which has been proposed to be among the fastest growing tissues known (Jablonka Shariff et al. 1993). The staining in the corpora lutea by BrdU-technique was localized to the vascular endothelium (Jablonka Shariff et al. 1993, Gaytan et al. 1996). The steroid producing cells in the corpora lutea were not stained. These two studies were performed in cycling rats and sheeps and did not describe the initial response to LH i.e. prior to ovulation. Incorporation of $^3$H-thymidine, as a marker of proliferation, was decreased in GCs 24h and 48h after hCG (LH-surge) stimulation (Rao et al. 1978) but the decrease was less pronounced in TCs. In paper IV, however, we can show an increase of PCNA in GCs as early as 2h after hCG-stimulation followed by a constant decrease during the first 24h (CL day 1). At 24h after hCG, PCNA was elevated in the residual ovary compared to corpus luteum , which could reflect the proliferation of non-
steroid producing cells as described before (Jablonka Shariff et al. 1993, Gaytan et al. 1996). A pronounced increase of c-Myc mRNA and protein was also observed 1h and 2h after hCG, respectively, both in GCs (Agarwal et al. 1996) (paper IV) and residual ovaries (paper IV). Suggesting that there is a proliferative response of the steroidogenic cells maybe concentrated to the TCs (Rao et al. 1978). However cyclin D2 protein decreased as early as 4h after hCG while cyclin E protein was not downregulated until 48 h after hCG (Robker et al. 1998a, 1998b). Interestingly, cyclin E was shown to be a downstream target of c-Myc (Leone et al. 1997, Bouchard et al. 1998), so the actual increased levels of cyclin E after hCG could be a result of the induction by c-Myc in GC. The cyclins and cell cycle progression are inhibited by i.e. p27Kip1. This protein was found in very low levels 4h after hCG and then increased to high levels 24h and 48h after hCG (Robker et al. 1998b). This high concentration of p27Kip1 might be the signal to effectuate a total stop in cell cycle mediated division of GCs.

**CCAAT/ENHANCER BINDING PROTEINS (C/EBPs) IN TUMORIGENESIS**

Originally detected in liver (Diehl 1998) and adipose (Darlington et al. 1998) tissues, the C/EBP transcription factor proteins have now been found in cells of several other origins (Lekstrom Himes et al. 1998) like breast (Robinson et al. 1998, Seagroves et al. 1998), lung (Flodby et al. 1996), ovary (Piontkewitz et al. 1993, Sirois et al. 1993), gastro-intestinal tract (Chandrasekaran et al. 1993, Blais et al. 1995) and hematopoetic cells (Zhang et al. 1997). Although the C/EBPs play a role in the acute phase response (Poli 1998), as they both can activate cytokines i.e. interleukin-6 (IL-6) and IL-8 or be activated by IL-6, I will in this section concentrate on C/EBPs role in regulation of the cell cycle.

As was discussed in the section of Specific Background the different proteins of the C/EBP family of transcription factors either take part in differentiation (C/EBPα) or proliferation (C/EBPβ and δ) or block transcription (C/EBPζ). The C/EBPs has actually been found to interact with several regulators of cell cycle progression. Firstly, C/EBPα was found to activate transcription of and stabilizing p21(WAF-1) protein (Timchenko et al. 1996, 1997). This protein is an inhibitor of cyclin-dependent kinase. Secondly, the retinoblastoma gene (RB), which is suggested to be a gatekeeper for entry into the S-phase of cell division, interact with C/EBP during adipocyte differentiation (Chen et al. 1996a). Thirdly, the proto oncogene protein c-Myc, that facilitate G1-progression of the cell cycle, inhibits or downregulates the expression of C/EBPα (Freytag et al. 1992, Antonson et al. 1995, Mink et al. 1996, Piontkewitz et al. 1996b). Moreover, cyclin D2, a cell cycle activating protein has consensus binding sites for C/EBPs in its promoter (Jun et al. 1997). These studies provide some clues to the earlier findings of C/EBPα as an activator of terminal differentiation in adipocytes (Samuelsson et al. 1991), since cell division can be halted through interaction with several known key regulators in the cell cycle.

In line with this C/EBPα should be downregulated or inactivated during tumor progression. In ovarian (paper V) and colorectal (Rask et al. 1999 unpublished results) tumors C/EBPα was found in all tumor samples and did not change with tumor progression. However, C/EBPα was localized to the cytosol of the cells, which might indicate that it was inactivated. In liver carcinomas C/EBPα was found in both nucleus and cytoplasm of the tumor cells and decreased in preneoplastic nodules (Flodby et al. 1995) and dedifferentiated tumors (Xu et al. 1994). Moreover, induction of C/EBPα demonstrated anti-proliferating functions i.e. growth arrest, in tumor cell-lines (Watkins et al. 1996).

During liver regeneration C/EBPβ and δ was found to be activated (Flodby et al. 1993). In fact, increased C/EBPβ DNA-binding activity during G1 phase of the hepatocyte cell
cycle was demonstrated in liver regeneration in vivo (Rana et al. 1995). A role for C/EBPβ in breast was also shown as normal lobuloalveolar proliferation during mammary gland development was disturbed in C/EBPβ -/- mammary glands (Robinson et al. 1998, Seagroves et al. 1998). Moreover, ovarian follicles from C/EBPβ -/- mice (Sterneck et al. 1997) or treated with antisense C/EBPβ (Pall et al. 1997), do not develop normally, have a markedly decreased ovulation rate and the C/EBPβ -/- mice have no corpus luteum. More recently another role for C/EBPβ was shown, as treatment of colorectal cancer cells with antioxidants could lead to apoptosis by induction of p21(Waf1) through a mechanism involving C/EBPβ (Chinery et al. 1997). Both the involvements C/EBPβ in cell proliferation and apoptosis are interesting in the aspect of tumorigenesis. This transcription factor has been found in the nucleus of ovarian (Paper V) and colon (Rask et al. 1999), unpublished results) cancer cells and increased during ovarian tumor progression in correlation with dedifferentiation and invasion. Interestingly, the shorter form of C/EBPβ (the LIP protein) was detected only in the malignant ovarian tumors (paper V). A specific increase of LIP was also found in human breast tumors and LIP was suggested as a marker for the identification of patients with poor prognosis (Zahnow et al. 1997). However, LIP is probably not functional as an activator of transcription since the activating domain in the N-terminal part of the protein is missing (Descombes et al. 1991). LIP can instead form inactive dimers with the full-length C/EBPβ (LAP) and the LAP/LIP ratio have been suggested to be of importance for the transcriptional activity (Raught et al. 1995).

Cancer invasion, that is, synthesis of proteolytic enzymes and transport through the extracellular matrix are thought to be an interplay between the cancer cells and the non-neoplastic stromal cells (Johnsen et al. 1998). The increased degradation of collagen in the extracellular matrix, which facilitates the migration of tumors cells, is mediated by matrix metalloproteinases (MMPs) (Shapiro 1998). The MMPs are predominantly produced by surrounding host stromal and inflammatory cells in response to factors released by tumors but also by the tumor cell itself (Shapiro 1998). In this aspect the recent finding that C/EBPβ induce transcription of the collagenase-1 (MMP-1) gene is very interesting (Doyle et al. 1997). MMP-1 seems to be the enzyme that is principally responsible for collagen turnover in most human tissues. The increased expression of C/EBPβ in ovarian tumor cells described in paper V, could in this way also be an activator of cancer invasion.

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REFERENCES


AGARWAL, P., PELUSO, J.J. and WHITE, B.A. Steroidogenic factor-1 expression is transiently repressed and c-myc expression and deoxyribonucleic acid synthesis are induced in rat granulosa cells during the periovulatory period. Biol Reprod., 55, 1271-1275 (1996).


DARAI, E., SCOAZEC, J.-Y., WALKER-COMBROUZE, F., MLIKA-CABANNE, N., FELDMANN, G., MADELENAT, P. and POTET, F. Expression of cadherins in benign, borderline, and malignant ovarian epithelial


GUILFORD, P., HOPKINS, J., HARRAWAY, J., MCLeod, M., MCLeod, N., HARAWIRA, P., TAITE, H., SCOULAR, R., MILLER, A. and REEVE,


interleukin-8 - A cyst fluid marker for malignant epithelial ovarian cancer?


KAWANASHI, J., KATO, J., SASAKI, K., FUJI, S., WATANABE, N. and NIITSU, Y. Loss of E-cadherin-dependent cell-cell adhesion due to mutation


MORTON, R., A., EWING, C., M., NAGAFUCHI, A., TSUKITA, S., ISAACS, W. and B. Reduction of E-cadherin levels and deletion of the a-


NORDENSTRÖM, K. 1981. .


OYAMA, T., KANAI, Y., OCHIAI, A., AKIMOT, S., ODA, T., YANAGIHARA, K., NAGAFUCHI, A., TSUKITA, S., SHIBAMOTO, S., ITO, F., TAKEICHI, M., MATSUDA, H. and HIROHASHI, S. A truncated beta-catenin disrupts the interaction between E-cadherin and alfa-catenin: a


PEIFER, M. Regulating cell proliferation: as easy as APC. *Science.*, **272**, 974-975 (1996).


TANNER, B., HENGSTLER, J.G., LUCH, A., MEINERT, R., KREUTZ, E., ARAND, M., WILKENS, C., HOFMANN, M., OESCH, F., KNAPSTEIN, P.G. and BECKER, R. C-myc mRNA expression in epithelial ovarian carcinomas in relation to estrogen receptor status, metastatic spread, survival
time, FIGO stage, and histologic grade and type. *Int J Gynecol Pathol.*, 17, 66-74 (1998).


