ENDOMETRIAL CANCER

from a molecular genetic perspective

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Endometrial cancer

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Endometrium kanker moleculair genetisch bezien

Proefschrift

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"Cancer is essentially a genetic disease at the cellular level."
W.F. Bodmer

Aan mijn ouders.
Voor Edzko en Meint.

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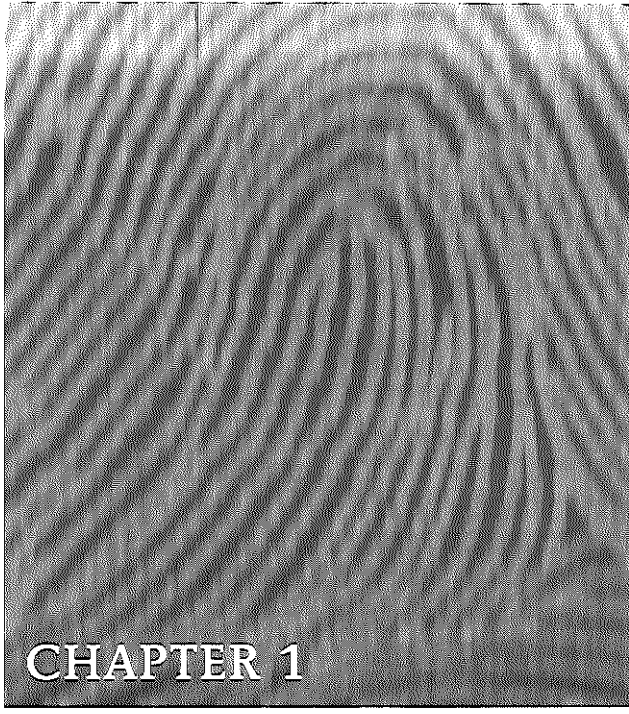
Abbreviations

ADAMTS-1	a disintegrin and metalloprotease with thrombospondin type1 motif
AF	activation function
AR	androgen receptor
Bmax	maximal number of binding sites
BUS	B-upstream segment
Carc	carcinoma
CBG	cortico-steroid-binding globulin
CBP	CREB-binding protein
CREB	cAMP response element-binding protein
DBD	DNA binding domain
DDC	gene deleted in colorectal carcinoma
(c)DNA	(complementary) deoxyribonucleic acid
E_2	estradiol
EC	endometrioid carcinoma
EGF	epidermal growth factor
EIC	endometrial intraepithelial carcinoma
(h)ER	(human) estrogen receptor
EST	expressed sequence tag
FAP	familial adenomatous polyposis
FIGO	International Federation of Gynecology and Obstetrics
FISH	fluorescence in situ hybridization
FKBP51	FK506 binding protein
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
GR	glucocorticoid receptor
GRIP-1	glucocorticoid receptor interacting protein-1
GTF	basal transcription factors
Gy	Gray
HAT	histone acetyltransferase
HD	histone deacetylation
hMLH1	human MutL homolog 1
hMSH2 (-6)	human MutS homolog 2 (-6)
HNPCC	hereditary non-polyposis colorectal carcinoma
hPMS2	human homolog of yeast MutL gen
HRE	hormone response element

hsp	heat-shock protein
Hyperpl	hyperplasia
ID	inhibitory domain
IGF	insulin-like growth factor
IGFBP3	insulin-like growth factor binding protein 3
IK-par	parental Ishikawa cells
kb	kilo base
Kd	equilibrium dissociation constant
kDa	kilo Dalton
LBD	ligand binding domain
LDL	low density lipoprotein
LH	luteinizing hormone
LOH	loss of heterozygosity
MAP	microtubule associated protein
MI = MSI	microsatellite instability
MMR	DNA mismatch repair system
MPA	medroxyprogesterone acetate
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NR	nuclear receptor
P/CAF	p300/CBP-associated factor
PCR	polymerase chain reaction
PIC	pre-initiation complex
(h)PR	(human) progesterone receptor
(h)PRA	(human) progesterone receptor isoform A
(h)PRB	(human) progesterone receptor isoform B
PRAKO	PRA knockout
PSC	papillary serous carcinoma
Rb	retinoblastoma
RER	replication errors
RNA Pol II	RNA polymerase II
SCR-1	steroid receptor coactivator 1
TAM	tamoxifen
TGF	transforming growth factor
TSC-22	transforming growth factor-beta-stimulated clone-22



CHAPTER 1

Scope of this thesis

1.1 Introduction

The first observations indicative of a role of genetic factors in carcinogenesis were made as early as 1912, when Rous demonstrated that a filterable agent (i.e. virus) could induce cancer in chicken (Rous 1965). In 1914, Boveri postulated a "genetic" theory on carcinogenesis by hypothesizing that the development of malignant tumor cells is caused by either the predominance of chromosomes which promote cell division, or by the elimination of chromosomes which inhibit cell division (Boveri, 1914).

In the last decade, research techniques in molecular biology have advanced rapidly. As a result, biological science has recently made huge steps forward in understanding the human genome. The disclosure of the human genome seems imminent, as, in February 2001, two research groups (the Human Genome Project (HGP) and Celera Genomics) published their draft sequences of the near complete human genome (Lander, Linton et al. 2001; Venter, Adams et al. 2001). With the knowledge of the human genome sequence and new molecular research techniques it is now possible to monitor gene expression levels on a genomic scale. These new data promise to enhance the fundamental understanding of life at the molecular level.

As, in general, genetic alterations are thought to play a major role in tumor development and tumor progression ((Fearon and Vogelstein 1990); (Knudson 1993)), knowledge of molecular genetics seems essential in understanding the etiology and the biological behavior of cancer.

Endometrial cancer ranks among the most common gynecologic malignancies in the Netherlands and North America, alongside ovarian cancer (Bristow 1999). In the Netherlands, each year approximately 1300 new cases are diagnosed, compared to 1400 for ovarian cancer and 700 for cervical cancer (1995) (Statistics Netherlands Ministry of Health, Welfare and Sports, 1999). In the majority of cases, approximately 75%, the tumor is confined to the uterus at the time of diagnosis and has a relatively good prognosis. This has let to the general perception of endometrial cancer to be a relative benign cancer type. However, in the Netherlands 370 women still die of endometrial cancer every year, compared to 234 of cervical cancer and 986 of ovarian cancer (in 1997) (Statistics Netherlands Ministry of Health, Welfare and Sports, 1999).

In order to predict the prognosis and to select optimal therapeutical re-

gimes, endometrial carcinomas are divided into subgroups. A surgical and histological staging system of the International Federation of Gynecology and Obstetrics (FIGO) (Pecorelli, Benedet et al. 1999) (http://www.figo.org) is at the present time the standard. However, 17-25% of the endometrial cancer deaths are accounted for by patients with low surgical-pathological stage (stage 1 and 2) whom are therefore misguidedly taken to have a good prognosis (Abeler and Kjorstad 1991). Patients with advanced/recurrent disease count for the other part of the deaths. In these patients response rates to treatment (surgery combined with radiotherapy) is low, indicated by a five-years survival rate of only 26% (Abeler and Kjorstad 1991). Hormone therapy (progesterone) or chemotherapy is given to these patients as a palliative treatment, however also with low response rates of only 10-30% (Lentz 1994; Rose 1996).

To improve the outcome of patients with endometrial cancer two strategies can be followed: improvement of *tumor classification* in order to facilitate a more accurate estimation of individual prognosis and tailored therapeutic options; improvement of treatment by identifying *new therapeutic targets and regimes.*

This thesis concerns itself with the possible role of molecular genetics with regard to these two strategies. The studies undertaken were designed to answer four questions:

Tumor classification

In general, tumor development and tumor progression are thought to be driven by genetic alterations. Integration of factors related to the biological behavior of the tumor, such as gene expression profiles, seems promising and may lead to a more accurate classification system of cancers. In this thesis the value of gene expression profiling in classifying human endometrial tumors was studied, answering the following question:

1. *What degree of clinical utility and subsequent diagnostic value of gene expression profiling of endometrial tumors can be ascertained?*

New therapeutic targets and regimes

Progesterone has a profound effect on mature human endometrium. In a

complex interaction with estradiol, progesterone controls proliferation and differentiation of endometrial epithelium during the menstrual cycle (Martin, Das et al. 1973). In clinical practice, progesterone is used as a palliative management of advanced and recurrent endometrial cancer. However, the response-rate of progesterone treatment in these patients is not very high (10-15%) (Lentz 1994; Rose 1996). The low response rate likely stems from the transition from hormone controlled growth towards hormone independent growth in advanced endometrial tumors. Understanding of the molecular mechanism underlying progesterone-induced growth inhibition of endometrial cancer cells and identification of genes responsible for the growth inhibiting properties of progesterone, may help to find tools to restore the progesterone-induced growth inhibition of endometrial cancer and subsequently lead to new therapeutic targets. In this respect thi thesis addresses the following questions:

2. *Is it possible to identify genes that are differential expressed between progesterone-sensitive and progesterone-insensitive endometrial cancer cells?*
3. *What functional differences between the two human progesterone receptor (hPR) isoforms (hPRA and hPRB) in endometrial cancer cells can be established?*
4. *What effects of progesterone can be measured on gene expression and cell growth of endometrial cancer cell-lines expressing hPRA or hPRB or both?*

1.2 Outline of this thesis

In **Chapter 2** the biological and clinical characteristics of endometrial cancer are described. An overview is presented on genes possibly involved in endometrial cancer development. Chapter 2 also describes the biological characteristics of progesterone and its receptors.

In **Chapter 3** gene expression profiles of human endometrial cancer tissue samples were obtained using a cDNA expression array technique. Subsequently, a method to analyze the gene expression data was established.

In **Chapter 4** the clinical utility and the diagnostic value of gene expression profiling in endometrial cancer tissue samples was studied.

In **Chapter 5** genes were identified which are differential expressed between progesterone-sensitive and progesterone-insensitive endometrial cancer cells.

In **Chapter 6** functional differences between the two human progesterone receptor (hPR) isoforms (hPRA and hPRB) in endometrial cancer cells was studied.

In **Chapter 7** progesterone effects on gene expression and cell growth were studied in several different endometrial cancer cell-lines expressing hPRA or hPRB or both.

1.3 Model systems used in this thesis

Endometrial cancer tissue bank

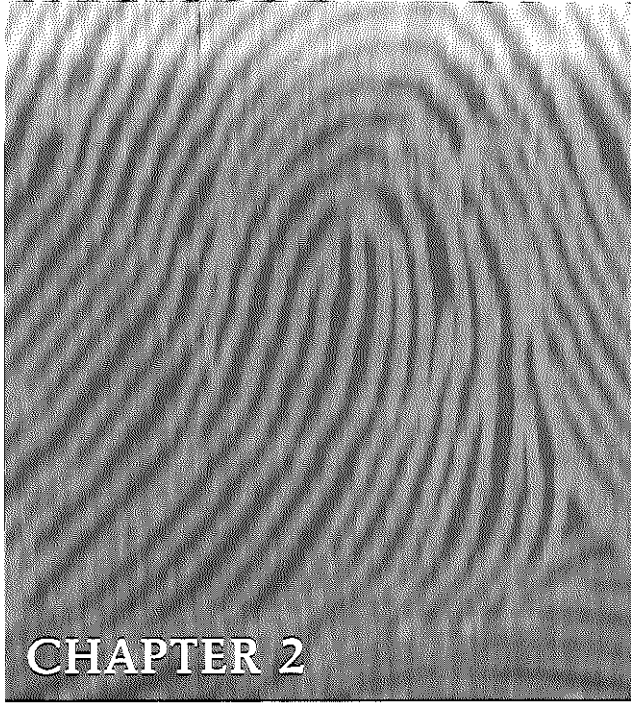
To establish the value of gene expression profiling in classifying human endometrial tumors, human endometrial tissue samples were used. An endometrial carcinoma tissue bank was established using hysterectomy specimen obtained from patients attending hospital[1] for treatment of endometrial cancer. Approval by the human subjects review boards was obtained in the participating hospital. Where possible, benign endometrial and myometrial samples were taken from the same patients. Benign endometrial samples from hysterectomy material of patients with a uterus myomatosis or menstrual bleeding disorders without a uterine carcinoma were furthermore collected. The age of the patient, the histological type and grade of the tumor and 5 years follow-up data were registered.

Endometrial cancer cell lines

To study the growth inhibiting properties of progesterone on endometrial cancer cells, several progesterone responsive endometrial carcinoma cell lines were constructed. Using stable transfection techniques, both the human progesterone receptor isoforms (hPRA and hPRB) were reintroduced into a well-differentiated human endometrial cancer cell line (a hPR negative sub-clone of the Ishikawa cell line). Several Ishikawa sub-cell lines were

[1] Department of Obstetrics & Gynecology of the Erasmus MC Rotterdam and department of Obstetrics & Gynecology of five affiliated hospitals: Saint Franciscus Hospital Rotterdam, Medical Center Rijnmond Zuid Rotterdam, Ikazia Hospital Rotterdam, Reinier de Graaf Group Delft and Albert Schweitzer Hospital Dordrecht.

constructed, each expressing different levels of either hPRA, or hPRB or both hPRA and hPRB.



General Introduction

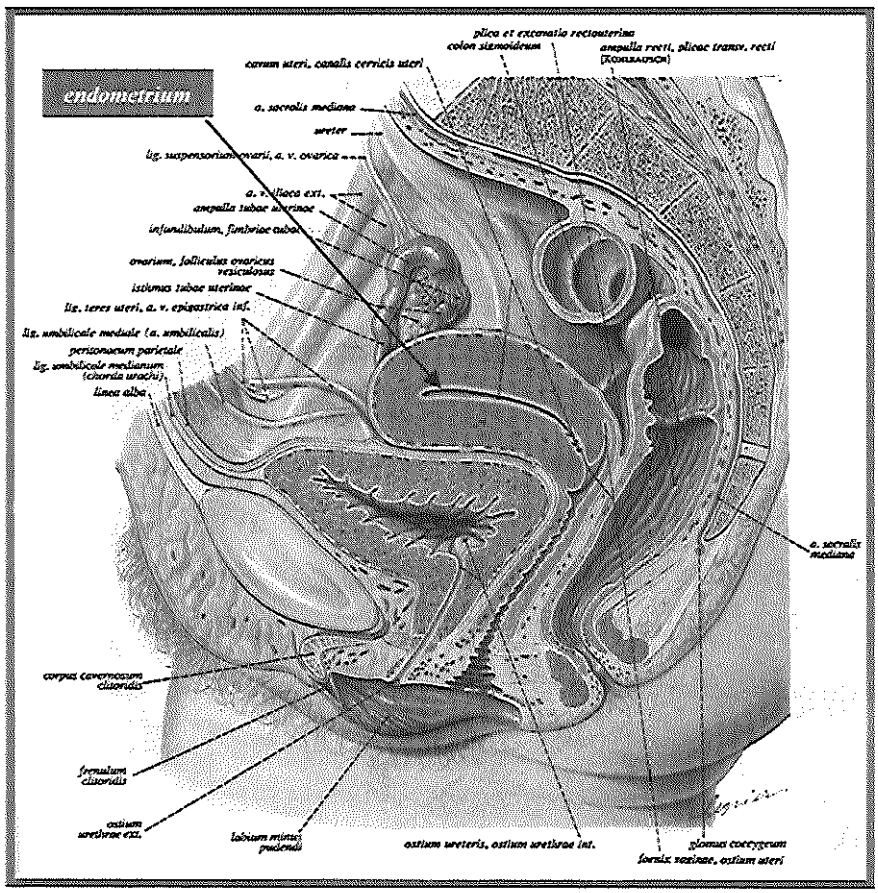


Figure 2.1.1: Sagittal section of the pelvis of an adult woman.
(copy of figure 297 page 203 of Sobotta Atlas of Human Anatomy, 10th edition 1982, Urban & Scharzenberg, with permission)

2.1 Endometrium and endometrial cancer

2.1.1 The normal endometrium

Morphology of the endometrium

The endometrium is the inner layer of the uterus, covering the uterine cavity (Figure 2.1.1). During the menstrual cycle it undergoes extensive morphological changes in order to prepare for implantation of the embryo, in case fertilization would occur. The endometrium consists of two distinct layers or zones, which differ from each other both in histological characteristics and in function: a basal layer and a functional layer. The *basal layer* is in direct contact with the myometrium, and undergoes little change throughout the menstrual cycle. During menses the basal layer stays intact. The *functional layer* surrounds the lumen of the uterine cavity and changes extensively throughout the menstrual cycle (figure 2.1.2). With every menstrual flow this layer is been sloughed. Afterwards, the new functional layer arises from the basal layer. The functional layer of the endometrium exists of two differ-

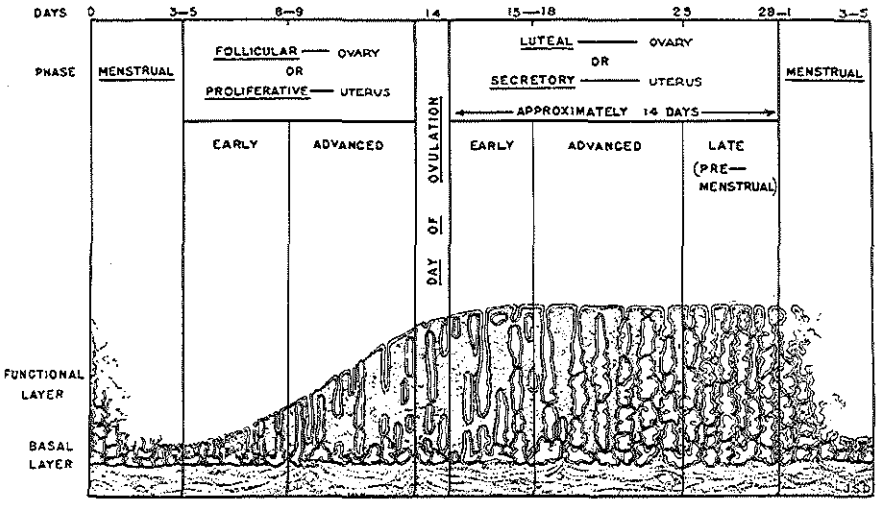


Figure 2.1.2: Cyclic changes of the endometrium. Cyclic changes in thickness and in form of glands and arteries of the endometrium and the relation of these changes to those of the ovarian cycle (from Williams Obstetrics Appleton&Lange 1993 p95, with permission).

ent cell-types: the glandular cells, which form the endometrial glands and cover the surface of the endometrium, and the surrounding stromal cells (Ludwig & Spornitz, 1991).

The blood supply of the endometrium consists of a network of arterial and venous channels. The arteries responsible for the blood supply of the endometrium, the spiral arteries, arise within the myometrium from branches of the uterine artery, subsequently pass through the basal endometrial layer, and extend into the functional zone. The proximal portion of these spiral arteries distributes blood to tissues of the basal layer and is not influenced by hormonal changes. However, the distal part of the spiral arteries, distributing blood to the functional layer, undergoes cyclic regeneration and degeneration during each menstrual cycle in response to hormonal changes.

Hormonal regulated changes of the normal endometrium

The endometrial cycle can be subdivided into three major phases: the proliferative or follicular phase, the secretory or luteal phase and the menstrual phase (Figure 2.1.2). During the preovulatory or proliferative phase estradiol stimulates proliferation and gland formation as well as vascular growth in the functional layer of the endometrium. Moreover, estrogens exhibit a pro-inflammatory effect on the endometrium with an influx of neutrophils and macrophages and stimulation of tissue edema (De & Wood, 1990; Kachkache et al., 1991; Quarmby & Korach, 1984). After the LH/FSH surge and the subsequent ovulation, progesterone is secreted by the corpus luteum, resulting in an increase of serum progesterone levels and the start of the secretory phase (Baird et al., 1975; McNatty et al., 1979). During this phase, progesterone stimulates differentiation of the glandular cells. Glycogen-rich vacuoles are formed in the glandular cells, which subsequently secrete their contents into the glandular lumen. In the stroma, progesterone induces pseudodecidual changes (Song & Fraser, 1995). Figure 2.1.3 illustrates these morphologic changes, correlated with the day of the menstrual cycle. In the absence of pregnancy, the corpus luteum regresses and the serum levels of estradiol and progesterone will drop (McNatty et al., 1979). In response to these hormonal changes, the spiral arteries in the endometrium constrict, leading to stasis, tissue ischaemia and sloughing of the functional layer of the endometrium (Noyes et al., 1950).

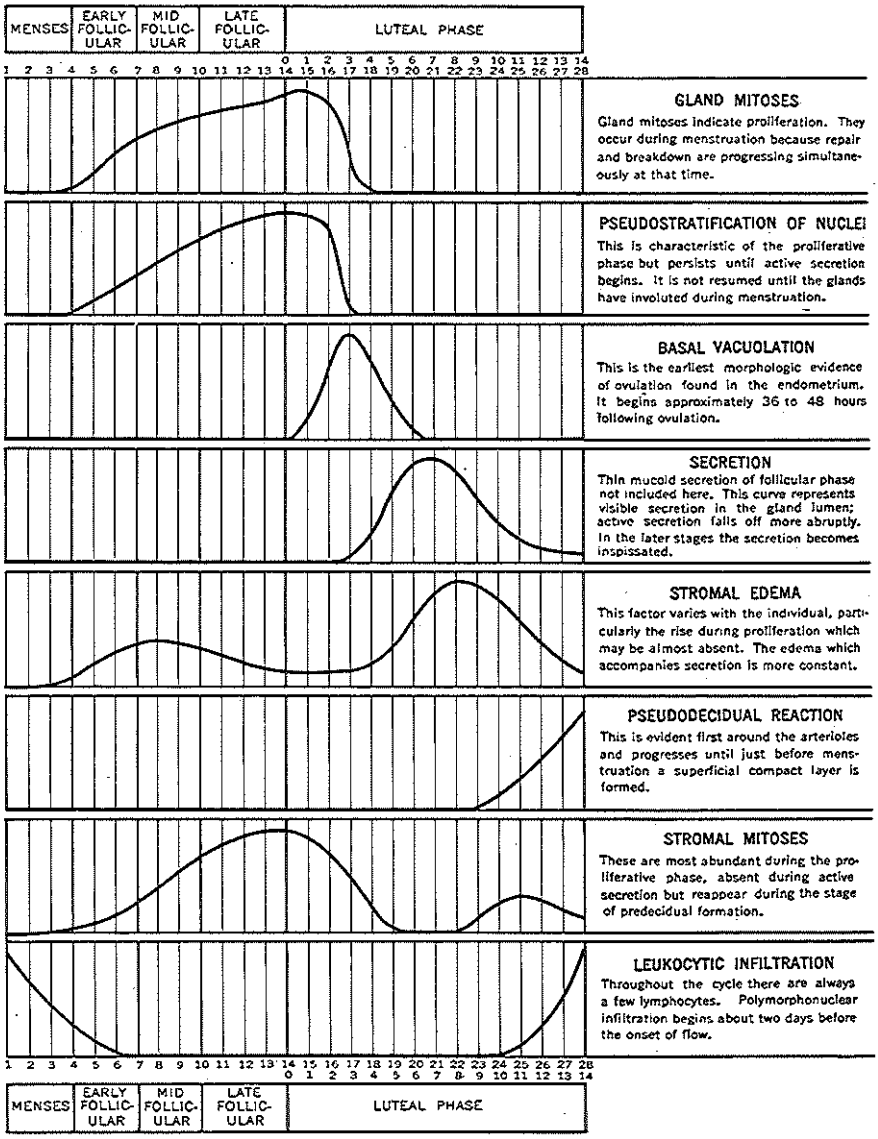


Figure 2.1.3: Morphological changes of the endometrium. Typical morphological findings correlated with the day of the menstrual cycle during a hypothetical 28-day ovarian cycle (from Noyes et al., 1950 with permission)

Progesterone effects on the endometrium

Progesterone has been observed to antagonize the proliferative and pro-inflammatory activity of estrogen on the human endometrium and subsequently causes gland differentiation, with glycogen secretion, stromal decidualization and suppression of immune responses (Song & Fraser, 1995; Clemens et al., 1979; Siiteri et al., 1977). These observations have been confirmed in progesterone receptor (PR) knockout mice, in which endometrial epithelium hyperplasia, stromal hypocellularity, and uterine inflammation has been observed (Lydon et al., 1996; Tibbetts et al., 1999). Other effects of progesterone include inhibition of uterine contractions, increased viscosity of cervical mucus, and extra-uterine effects such as glandular development of the breast, and increase in basal body temperature (Humphreys et al., 1997; Lofgren & Backstrom, 1994; Stachenfeld et al., 2000). Prolonged stimulation of the endometrium with progestins lead to glandular atrophy and stromal necrosis, resulting in an atrophic endometrium (Ludwig, 1982; Song & Fraser, 1995).

2.1.2 Endometrial hyperplasia

Endometrial hyperplasia is an overgrowth of endometrial glands and endometrial stroma, and is characterized by a proliferative glandular pattern with varying degrees of atypia (Welch & Scully, 1977). Hyperplasia demonstrating atypia may develop into endometrial cancer (Kurman et al., 1985). Relative estrogen excess seems to induce hyperplasia. Women exposed to excess of estrogens such as in polycystic ovarian syndrome, estrogen-secreting ovarian tumors, obesity and unopposed exogenous estrogens are at risk (Kreiger et al., 1986; Baanders-van Halewyn et al., 1996). Atypical hyperplasia is treated with progestins to antagonize the estrogenic effects or with surgery (hysterectomy). Without treatment, patients with atypical hyperplasia have a 25% risk of developing endometrial carcinoma (Kurman et al., 1985).

2.1.3 Endometrial cancer

Incidence and mortality

Endometrial cancer ranks as the most common gynecologic malignancy in Europe and North America, alongside ovarian cancer. It was estimated that in the United States 37,000 new cases and 6400 deaths occur per year

(Bristow, 1999). In the Netherlands, the incidence of endometrial cancer for 1995 has been set on 1337 cases, compared to 1447 for ovarian cancer and 706 for cervical cancer. In 1997, 370 women died of endometrial cancer compared to 234 of cervical cancer and 986 of ovarian cancer (reported by the Central Office of Statistics, Ministry of Public Health, The Netherlands, 1999). The highest incidence was found in the age category 65-85 years.

Histopathologic classification of endometrial cancers

The majority of endometrial cancers arises from glandular cells and is known as *adenocarcinoma,* which can be subdivided into endometrioid and non-endometrioid adenocarcinoma. Stromal cells of the endometrium can become malignant and develop into *endometrial stromal sarcomas,* however this is a rare event. Sometimes a combination of stromal sarcoma and adenocarcinoma is observed and these endometrial tumors are typed as *mixed carcinosarcoma.* According to the WHO/ISGP (World Health Organization/International Society of Gynecological Pathology) histopathologic classification, endometrium carcinomas can be divided into different subgroups, which are shown in Table 2.1.1. Endometrioid adenocarcinoma is the most common form of endometrium carcinoma (90%) (Platz & Benda, 1995), followed by papillary serous adenocarcinoma (3-10%) (Bristow, 1999; Nicklin & Copeland, 1996). Other histopathologic subtypes of endometrial cancer are very rare.

Etiology

The etiology of endometrial cancer has been suggested to differ between the endometrioid and the non-endometrioid adenocarcinoma (Sherman, 2000). Endogenous (anovulation) and exogenous (hormonal therapy) sources of estrogen are well accepted as a cause for increasing the risk of endometrioid adenocarcinoma (Antunes et al., 1979; Gordon et al., 1977). Moreover, the risk factors linked to endometrial cancer are associated with an excess of estradiol. Endometrial cancer risk factors include: obesity, polycystic ovary disease, estrogen-producing ovarian tumors, early menarche and late menopause, nulli-parity, pure estrogen containing oral anticonceptiva or hormonal substitution, and diabetes mellitus (Rose, 1996). Excess of estrogens, without the differentiating effects of progesterone, are thought to give rise to atypical endometrial hyperplasia, which can develop into endometrioid adenocarcinoma (Lindahl & Willen, 1991; Kurman et al., 1985). Non-endometrioid adenocarcinoma, on the other hand, shows no association with

Table 2.1.1: FIGO Staging < 1988

Stage 0	Carcinoma in situ. Histological findings suspicious of malignancy.
Stage I	The carcinoma is confined to the corpus.
Stage Ia	The length of the uterine cavity is 8 cm or less.
Stage Ib	The length of the uterine cavity is greater than 8 cm
It is desirable that Stage 1 cases be sub-grouped with regard to the histological type of the adenocarcinoma as follows:	
	G1 Highly differentiated adenomatous carcinoma.
	G2 Differentiated adenomatous carcinoma with partly solid area.
	G3 Predominantly solid or entirely undifferentiated carcinoma
Stage II	The carcinoma has involved the corpus and the cervix.
Stage III	The carcinoma has extended outside the uterus but not outside the pelvis.
Stage IV	The carcinoma has extended outside the pelvis or has obviously involved the mucosa of the bladder or rectum

(FIGO staging endometrial cancer 1971-1988, (Mikuta, 1993))

unopposed estrogen exposure or with endometrial hyperplasia. Endometrial intraepithelial carcinoma (EIC), in which epithelium cells show high grade nuclear atypia, is often seen in non-endometrioid adenocarcinoma and is rare in endometrioid adenocarcinoma (Ambros et al., 1995).

The use of the anti-estrogen Tamoxifen (TAM) has been associated with an increased risk of endometrial cancer (Bergman et al., 2000). TAM is successfully used in the management of women with breast cancers. However, long term use of TAM gives a 2-7 fold increase of the relative risk for endometrial cancer (Bergman et al., 2000; Bernstein et al., 1999). The endometrial tumor, which develops in these patients, appears to be associated with the non-endometrioid histological type, high stage, and with low expression of the estrogen receptor (Bergman et al., 2000; Ramondetta et al., 1998; Kuwashima et al., 1998).

So far, a genetic predisposition for endometrial cancer has only been shown for members of families with HNPCC (**H**ereditary **N**on-**P**olyposis **C**olorectal **C**arcinoma) (Mecklin & Jarvinen, 1991). Women carrying mutations in the DNA repair genes known to be associated with HNPCC (e.g. hMSH2, hMLH1, hPMS2 and hMSH6 gene) are found to have a 22-43% lifetime risk of developing endometrial cancer, which is significant higher than the lifetime risk in the general Dutch or USA female population (1.7-3.3%)

Table 2.1.2: FIGO Staging ≥ 1988

Histopathologic classification:

- Endometrioid carcinoma
  - adenocarcinoma
  - adenocanthoma
  - adenosquamous carcinoma
- Mucinous adenocarcinoma
- Papillary serous adenocarcinoma
- Clear-cell carcinoma
- Adenosquamous carcinoma
- Undifferentiated carcinoma
- Mixed carcinoma

Histological grade (G)

GX	Grade cannot be assessed	
G1	Well differentiated	≤ 5% non-squamous or non-morular solid growth pattern
G2	Moderate differentiated	6-50 % non-squamous or non-morular solid growth pattern
G3	poorly or undifferentiated	≥ 50 % non-squamous or non-morular solid growth pattern

Surgical staging (Stage)

Ia	Tumor limited to the endometrium
Ib	Invasion to less than half of the myometrium
Ic	Invasion equal to or more than half of the myometrium
IIa	Endocervical glandular involvement only
IIb	Cervical stromal invasion
IIIa	Tumor invades serosa of the corpus uteri and/or adnexa and/or positive cytological findings
IIIb	Vaginal metastases
IIIc	Metastases to pelvic and/or para-aortic lymph nodes
IVa	Tumor invasion of bladder and/or bowel mucosa
IVb	Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes

((Pecorelli et al., 1999), http://www.figo.org)

(Watson & Lynch, 1993; Aarnio et al., 1995; Dunlop et al., 1997; Visser et al., 1998).

Diagnosis and screening

Endometrium tumors are usually diagnosed at an early stage, as the disease

presents itself with an alarming early symptom: abnormal vaginal bleeding. Endometrial-biopsy is used to diagnose endometrial cancer. Recently, ultrasonography has been demonstrated to exhibit diagnostic value in the triage of patients with postmenopausal bleeding (Gull et al., 2000; Kinkel et al., 1999; Karlsson et al., 1995). When endometrial-biopsy is not conclusive, endometrial-curettage sampling and hysteroscopy is performed to confirm or exclude the diagnosis

Staging and prognostic factors

Endometrial carcinomas are divided into subgroups in order to create a system to predict the prognosis and to select optimal therapeutic regimes. Generally, the FIGO (International Federation of Gynecology and Obstetrics) staging system is used. Before 1988 the FIGO staging used clinical criteria; including fractional curettage and pelvic examination, to stage endometrial tumors (Table 2.1.1). As more information became available with regard to risk factors associated with natural behavior of endometrial cancer and survival, the staging system changed. Identified as risk factors were histological grade, depth of myometrial invasion, extension into the cervical canal, vascular space invasion, pelvic node metastases, aortic node metastases, adnexal metastases, penetration of uterine serosa and positive peritoneal cytological findings. In 1988 a new FIGO staging system was introduced, including these risk factors. This new staging system assumes that most patients will be treated by a primary surgical approach and the staging is performed after surgery and is based on the surgicopathological findings (Table 2.1.2). If radiation has been applied prior to surgery, the former clinical staging system is used.

The surgical staging, as described by the FIGO staging system of 1988, includes lymph node sampling (lymphadenectomy) and pelvic washing or samples of ascitic fluid. However, no consensus has been established on the value of lymphadenectomy, neither in the staging process nor as a part of the treatment (Podratz et al., 1998). As a result, in several clinics and some countries, lymphadenectomy is not used as a standard in staging endometrial cancers.

The FIGO staging system criteria have significantly changed between the periods prior to and after 1988. However, the nomenclature in the two systems is identical. Therefore, when comparing research data involving FIGO-staged endometrial cancers knowledge of the date of the staging system used

is essential. In a Norwegian population-based study, the five-years survival rates for the different staging groups were established using both the FIGO clinical staging system and a 'surgicopathological' staging system (not further specified by the authors) (Abeler & Kjorstad, 1991). They found the five-years survival rate to be: clinical Stage I = 83%, clinical Stage II = 73%, clinical Stage III = 52% and clinical Stage IV = 27%, compared to surgicopathological Stage I = 83%, surgicopathological Stage II = 75%, surgicopathological Stage III = 41% and surgicopathological Stage IV = 26%.

Several studies have shown that the prognosis of patients with endometrial cancer is correlated with various factors other than FIGO stage. Morphometric nuclear grade (Salvesen et al., 1998), lymph-vascular space invasion (Feltmate et al., 1999), DNA ploidy (Evans & Podratz, 1996; Lim et al., 1999), estrogen/progesterone receptor status (Creasman, 1993) and p53 overexpression (Soong et al., 1996) have been shown to be independent prognostic factors. Evaluating the prognosis by integrating FIGO staging system with factors related to the biological behavior of the tumor seems promising. However, a useful model has yet to be established.

Therapy

Surgery is the primary treatment in the majority of women with endometrial cancer. Radiotherapy is generally used when adjuvant therapy is indicated. However, indication for adjuvant therapy differs around the world. Chemotherapy and hormonal treatment are only given in clinical trial contexts or as palliation in case of an advanced disease. The FIGO staging and the health condition of the patient generally guide the choice of treatment.

Stages I and II: Hysterectomy with bilateral salpingo-oophorectomy has a high curation rate in patients with a stage I/II disease (Cirisano et al., 2000). The value of adjuvant radiotherapy in stage I patients remains unclear. In a recent Dutch randomized trial including 715 patients from 19 radiation oncology centers (PORTEC Study Group, (Creutzberg et al., 2000)) patients with Stage-I endometrial carcinoma were randomized to pelvic radiotherapy (46 Gy) versus no further treatment, following total abdominal hysterectomy and bilateral salpingo-oophorectomy (without lymphadenectomy). From this study it was concluded that postoperative radiotherapy in Stage-I endometrial carcinoma reduces loco-regional recurrence but has no impact on overall survival. Moreover, radiotherapy increases treatment-related morbidity.

Stages III and IV: The cornerstone in treatment of these patients consists

of abdominal hysterectomy with bilateral salpingo-oophorectomy in combination with postoperative radiotherapy. In the presence of metastasis outside the pelvic area, chemotherapy or hormonal treatment with progestins is optional.

2.1.4 Hormone responsiveness of endometrial cancer

The normal endometrium is highly sensitive to hormonal influences. All morphological changes of the endometrium during the menstrual cycle are induced by the ovarian hormones estradiol and progesterone (discussed in paragraph 2.1.1). Also, a high responsiveness to hormonal treatment (e.g. progestins) is found for benign functional endometrial disorders, such as menorrhargy, metrorrhargy, endometriosis and endometrial hyperplasia (Lindahl & Willen, 1991). However, hormonal treatment (e.g. progestins) of patients with endometrial cancer (FIGO-Stage III/IV) results in low response rates of only 10-30% (Lentz, 1994). Even more, in a meta-analysis of six randomized controlled trials no association was found at all between progesterone treatment of endometrial cancer patients and reduction in relapse time or overall survival rate (Martin-Hirsch et al., 1996). However, no discrimination was made between different clinical stages or histological grades. Other studies have indicated that well differentiated tumors (expressing estrogen- and progesterone receptors) respond better than poorly differentiated tumors (Gurpide, 1981; Boman et al., 1993).

2.2 Progesterone and its receptors

2.2.1 Progesterone biosynthesis

Progesterone belongs to the group of steroid hormones. Other steroid hormones are estrogens, androgens, glucocorticoids and mineralocorticoids. Steroid hormones are mainly produced in steroidogenic glands (e.g. ovaries, testes and adrenals) and are secreted into the circulation. Some peripheral tissues can locally produce steroids from steroid precursors. Steroid hormones are important molecules, and are involved in regulating critical physiologic events such as cellular differentiation, growth and death.

A perhydro-1,2-cyclopentanophenanthrene-ring structure is the basic

carbon skeleton for all steroid hormones. The steroidogenic glands synthesize the steroids from cholesterol. These glands are able to synthesize cholesterol de novo from 2-carbon precursors (cholesteryl esters) but can also use cholesterol from circulating low-density lipoproteins (LDL). The 27-carbon cholesterol molecule is subsequently converted to pregnenolone by removal of a six-carbon fragment to form the 21-carbon steroid molecule. Pregnenolone serves as a precursor molecule from which all the steroids can be formed. Enzymatic reactions are needed to convert pregnenolone into different steroids. Several enzymes are involved in this process: 3β-hydroxysteroid-dehydrogenase for conversion of Δ5 to Δ4 steroids; 17α-hydroxylase/ C_{17-20} lyase enzyme systems for conversion of C_{21} to C_{19} steroids, 17β-hydroxysteroid-dehydrogenase, and aromatase activity for conversion of androgens to estrogens.

The biochemical pathways of steroid hormone biosynthesis, including the major enzymes, are similar in all steroidogenic glands. However, different cell types express different amounts of these enzymes, so that the predominant steroids produced differ in the various cells. Even within one gland, the different cell types may produce different steroid hormones. This phenomenon resulted in the two-cell theory for steroid hormone production in the ovary. The theca and granulosa cells of the ovarian follicles were thought to have a specific role in synthesis of steroids (Makris & Ryan, 1977). In this theory, the theca cells convert cholesterol to androstenedione and testosterone. Granulosa cells are particularly rich in aromatase activity and can convert androgens to estrogen. The androgens synthesized within the theca cells were thought to be transported to the granulosa cells where they can be used as substrate for estrogen production. In the luteal phase, the corpus luteum granulosa cells are thought to convert cholesterol to progesterone and 17α-hydroxyprogesterone. Later, McNatty et al. (McNatty et al., 1979) postulated that the ovarian steroid synthesis might not be so rigidly compartmentalized. Additional to the two-cell theory, the steroids secreted by the granulosa and theca cells are now thought to enter different cellular compartments before leaving the ovary. As a result, the different steroid-producing tissues of the follicle may exert their primary effects at different sites.

Gonadotropin hormones control the biosynthesis of steroids in the ovary. Luteinizing hormone (LH) acts primarily to regulate the first step in steroid hormone biosynthesis, namely the conversion of cholesterol to pregnenolone, and also induces subsequent enzymes in the pathway. In the follicular

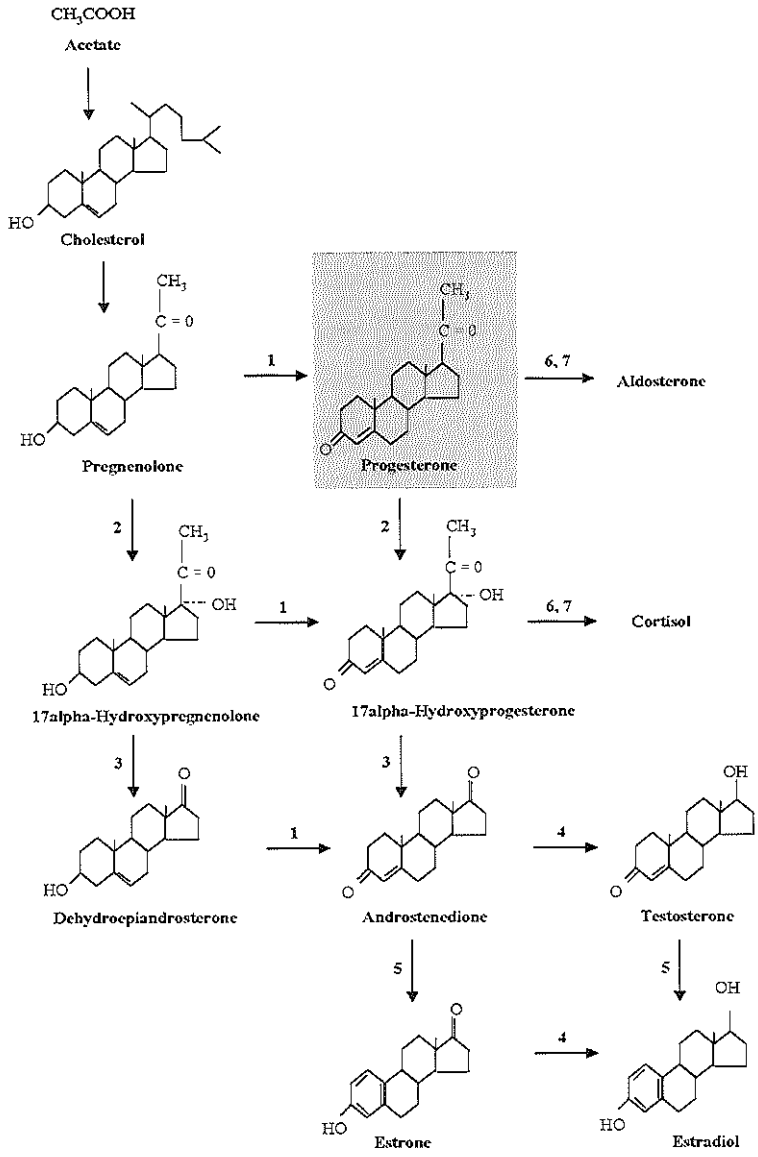


Figure 2.2.1: Steroid biosynthesis. Pathways for steroid biosynthesis involve several different enzyme systems. **Enzyme 1:** 3β-hydroxysteroid-dehydrogenase, **enzyme 2:** 17α-hydroxylase, **enzyme 3:** 17, 20-lyase, **enzyme 4:** 17β-hydroxysteroid-dehydrogenase, **and enzyme 5:** aromatase.

phase this results in stimulation of the production of androgens by theca cells. During the luteal phase, LH facilitates the production of progesterone by the corpus luteum (McNatty et al., 1980). In granulosa cells, aromatization of androgens into estrogens is regulated by follicle-stimulating hormone (FSH) (Sjogren et al., 1987), Montgomery Rice et al., 1998).

2.2.2 Secretion and metabolism of progesterone

The secretory activity of progesterone producing cells is closely coupled to

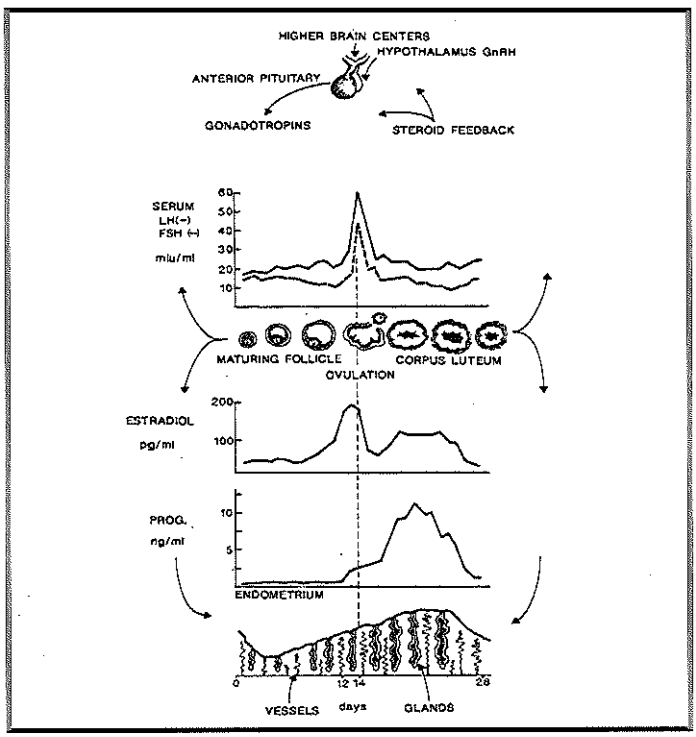


Figure 2.2.2: Changes in pituitary gonatropins FSH and LH, and ovarian hormones estradiol and progesterone throughout the menstrual cycle. Diagrammatic representation of the menstrual cycle showing the relationship and feedback system of the hypothalamic and pituitary hormones (gonadotropin-releasing hormone GnRH, FSH and LH) with the ovarian hormones (estradiol and progesterone), and the response of the endometrium to the change in steroid concentration. (from Kistner's Gynecology 6th edition, 1995, Mosby-year book, p13 figure 3-1, with permission)

their synthesis activity, since little is stored in the cells. When released into the circulation, progesterone is bound by plasma transport proteins. The most important transport protein for progesterone is cortico-steroid-binding globulin (CBG). Progesterone binds only weakly to albumin. The serum concentration of progesterone in women changes throughout the menstrual cycle (Figure 2.2.2).

During the follicular phase, ovarian progesterone production is low and circulating progesterone originates mainly from synthesis in the adrenal gland or from conversion of circulating precursors in peripheral tissues. After ovulation, the corpus luteum produces high levels of progesterone. In case of pregnancy, the syncytiotrophoblast takes over the progesterone production from the corpus luteum after 6-8 weeks. The trophoblast utilizes maternal plasma low-density lipoprotein (LDL) cholesterol to synthesize progesterone, as de novo synthesis of cholesterol is limited in these cells. Progesterone produced by the trophoblast is released into the maternal circulation, giving rise to increasing maternal serum levels of progesterone during pregnancy. The trophoblast also produces pregnenolone, which is released into the fetal circulation. The fetal liver and adrenals subsequently convert pregnenolone into other steroids.

Progesterone is cleared from the circulation by conversion into pregnanediol and by glucuronidation in the liver. In this process 20α-hydroxyprogesterone, a metabolite of progesterone, is formed which can act as progestagin, although with only 20% of the activity of progesterone. The end-product pregnanediol glucuronide is excreted in the urine.

2.2.3 The progesterone receptors

The family of steroid hormone receptors:
Progesterone mediates its function via a specific intracellular receptor, the progesterone receptor (PR). There exist two functional progesterone receptor isoforms (PRA and PRB), which are closely related to other steroid receptors, such as the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), the androgen receptor (AR), and to a lesser extent, the estrogen receptor (ER) (Evans, 1988). These steroid hormone receptors act as hormone-dependent transcription regulators and belong to the superfamily of nuclear receptors (NR).

Progesterone receptor gene, mRNAs and proteins:
The human progesterone receptor protein exists as two isoforms hPRA and hPRB (Horwitz & Alexander, 1983). The hPRA is a truncated form of the hPRB and lacks the first 164 N-terminal amino-acid residues. The two isoforms are transcribed from the same gene. Originally, the two isoforms were thought to be the result of alternative translation initiation from two in-frame ATG codons from the same mRNA, producing a PRA-protein of 94 kDa and a PRB-protein of 114 kDa (Conneely et al., 1987). However, transfection experiments have shown that vectors containing both the first and the second ATG codon produce exclusively the hPRB form (Kastner et al., 1990). The hPRA is generated from vectors containing only the second ATG. These findings argue against the hypothesis that the two isoforms are the product of alternative translation initiation and suggest that hPRA and hPRB are generated as a result of transcription from different promoters. Subsequently,

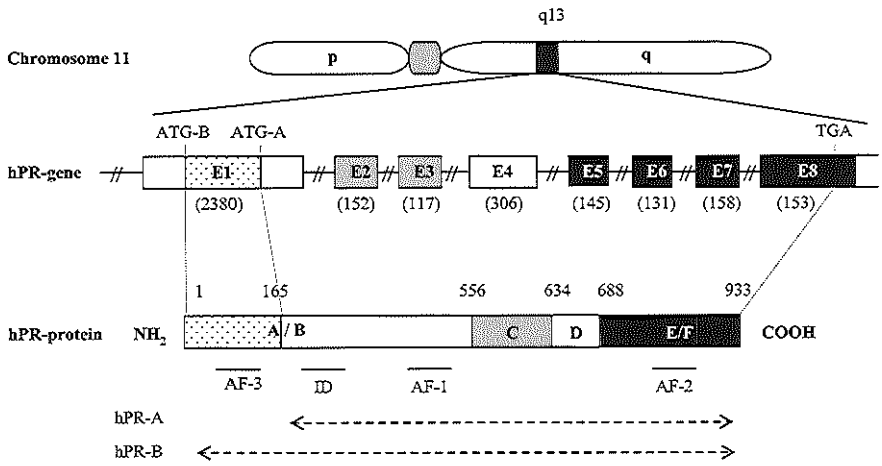


Figure 2.2.3: The human progesterone receptor gene (hPR): locus and protein. The hPR-gene was mapped to the long arm of chromosome 11 (Rousseau-Merck et al., 1987). The hPR protein is encoded by 8 exons (E1-E8) (Misrahi et al., 1993). Two hPR isoforms, hPRA (94 kDa) and hPRB (114 kDa), are being translated from two different transcripts. Both proteins contain four functional domains: NH_2-terminal domain (A/B), DNA-binding domain (C), hinge region (D) and ligand binding region (E/F). Three sub-domains with a transcription activation function (AF) have been identified (AF-1, AF-2, AF-3) and one sub-domain with a transcription inhibitory function (ID).

Kastner et al (Kastner et al., 1990) identified distinct promoters, initiating transcription of two different mRNAs. Moreover, exon deletions, in either hPRA or hPRB, have been found to give rise to new hPR-protein variants (Wei et al., 1990; Graham et al., 1995; Richer et al., 1998; Hirata et al., 2000; Leygue et al., 1996).

Except for the first 164 N-terminal amino-acids, both hPRA and hPRB proteins are structural identical (Figure 2.2.3). Both forms have similar DNA and ligand binding affinities (Lessey et al., 1983) and both forms mediate progesterone-activated gene transcription regulation. However, the transactivation function of hPRA and hPRB appear to differ between cell types and target gene promoters used (Tora et al., 1988; Vegeto et al., 1993; Hovland et al., 1998).

Structure of the progesterone receptors

The progesterone receptor structure is similar to that of the other steroid receptors in that it contains four discrete functional domains, an NH_2-terminal domain, a DNA-binding domain (DBD), a hinge region and a ligand or hormone binding COOH-terminal domain (LBD) (Mangelsdorf et al., 1995; Katzenellenbogen & Katzenellenbogen, 1996). The DBD is the most conserved among the steroid receptors, and the NH_2-terminal domain is the most variable region (Meyer et al., 1990).

- *NH_2-terminal domain* — The NH_2-terminus (domain A/B) is the least conserved domain among the steroid hormone receptor family. Transcription activation of target genes has been shown to depend on sub-domains with an activation function (AF). Deletion mapping has identified two specific AFs for all members of the steroid hormone receptor family. One ligand-independent activation function (AF-1) is located within the NH_2-terminus and the other, a ligand-dependent AF (AF-2) is found in the LBD. The location of the AF-1 and the amino acid composition of the NH_2-terminus differ significantly among the steroid hormone receptor family members. This seems to indicate that the NH_2-terminus is essential in receptor-specific transcription regulation. In the hPR, the NH_2-terminus contains a third activation function AF-3, which is constitutively active. This AF-3 is located in the 164 amino acid B-upstream segment (BUS), which is unique to hPRB (Giangrande et al., 1997). The hPRA isoform lacks this BUS, and consequently AF-3. An inhibitory domain (ID) has

been identified within the first 140 amino acid residues of the hPRA, and was show to prevent hPRA from functioning as a transcriptional activator and seems to contribute to the function of hPRA as a dominant repressor of steroid receptor transcriptional activity (Giangrande et al., 1997). Deletion of ID from hPRA resulted in a receptor mutant that is functionally indistinguishable from hPRB (Giangrande et al., 1997).

- *DNA-binding domain* — The DNA binding domain (domain C) is located in the center of the receptor molecule and consists of two zinc-clusters. The DBD binds to specific DNA sequences, hormone response elements (HREs), located in or near regulatory regions of target genes (Beato, 1989; Bagchi et al., 1988). The DBD is thought to fold into so-called "zinc-clusters" and only a very limited number of amino-acid residues within one of the α helixes of the first zinc cluster are responsible for specific recognition of the HRE's (Freedman et al., 1988; Mader et al., 1989). Binding of the DBD to the HRE results in activation or repression of gene transcription (Beato, 1989; Tsai & O'Malley, 1994). Both direct interaction of the DBD domain with the HRE, as interaction of receptor protein complex with other regulatory proteins are involved in this mechanism of gene transcription regulation (Ing et al., 1992).
- *Hinge region* — The hinge region (domain D), located between the DBD and LBD domain, is a poorly conserved region among the steroid hormone receptor family members (Evans, 1988). The hinge region contains a nuclear localization signal, and in the PR it is thought to be involved in homodimerization of the receptor, together with the LBD and the NH_2-terminal domain (Tetel et al., 1997).
- *Ligand binding region* — The LBD region (domain E/F) is the COOH-terminal part of steroid receptors. Apart from ligand binding, this region is also involved in receptor dimerization and transcription regulation. Ligand binding induces receptor dimerization and creates an 'active surface' on the LBD, exposing a transcriptional activating function (AF-2) (Webster et al., 1988; Kumar & Chambon, 1988; Tora et al., 1989). Ligand free LBD prevents the DBD from binding to HRE and therewith excludes transcription activation. Deletion experiments have shown that steroid receptors lacking a LBD act as constitutively active receptors.

Subcellular localization of the hPRs

The steroid hormones are synthesized in the cytoplasm. The hinge region

of the receptor contains a nuclear localization signal, directing the receptor towards the nucleus (Ylikomi et al., 1992). For the hPR, a shuttle model has been proposed, in which the receptor enters the nucleus in an energy-dependent manner, and may freely diffuse back into the cytoplasm (Guiochon-Mantel et al., 1991). However, the hPR appears to be predominantly located in the nucleus, even in absence of ligand (Gasc et al., 1989).

Transcription activation route:
The mechanism of action of PR is similar to that of other steroid receptors. In the absence of ligand, receptors are sequestered in a large complex of heat shock proteins (hsp90, hsp70 and hsp56) together with other proteins (Smith et al., 1990; Pratt & Toft, 1997). In the complex, the steroid hormone receptor is directly bound to hsp 90 and folded in a specific conformation, in which the receptor is unable to bind to DNA but capable of receiving and responding to hormonal signals (Smith, 1993). Upon hormone binding, the receptor undergoes a distinct change in conformation (DeMarzo et al., 1991), resulting in the dissociation of a monomeric receptor from the heat shock complex (Renoir et al., 1990). The receptor dimerizes and becomes phosphorylated. Subsequently, the receptor dimer binds to DNA on specific hormone response elements (HRE). These HREs are located in or near regulatory regions of target genes (Beato, 1989; Bagchi et al., 1988). On binding to the HREs, the receptor complex can interact with other proteins and directly or indirectly regulate gene transcription.

Several models have been proposed to explain the mechanism of steroid hormone receptor regulation of gene transcription. In the center of transcription activation, basal transcription factors (GTFs) together with RNA-polymerase-II (RNA Pol-II) form a complex: the pre-initiation complex (PIC). Subsequently, the PIC can recruit several other transcription factors, such as the steroid hormones receptors. To start transcription, RNA Pol-II interacts with the TATA box sequence at the promoter site. In one model, direct interaction of steroid hormone receptors with the PIC has been proposed to stabilize this PIC assembly and thereupon enhance the transcription activity (Ing et al., 1992; Fondell et al., 1996; McEwan & Gustafsson, 1997). A second model suggested that the interaction of steroid hormone receptor with intermediary factors or co-activators enhances the assembly of a stable PIC (Horwitz et al., 1996; Voegel et al., 1996). Subsequently, several coactivators have been cloned, that associate with progesterone receptors, and enhance

the ability of the progesterone receptor to transactivate target genes (reviewed by Rowan & O'Malley, 2000). A third model involves the accessibility of DNA to transcription regulating factors. Genomic DNA is packed into nucleosomal structures (Candau et al., 1996), in which the double-helix DNA is wound around an octamer of histones. The nucleosomal structure of DNA prevents transcription factor binding and represses basal transcription activity (Svaren & Horz, 1996). Targeted histone acetylation loosens the nucleosomal structure and creates an access for transcription factors to their response elements in the target gene promoter regions. Some of the steroid receptor associated coactivators, such as steroid receptor coactivator 1 (SCR-1), coactivator CREB (cAMP response element-binding protein)-binding protein (CBP) and p300/CBP-associated factor (P/CAF), have been found to possess intrinsic histone acetyltransferase (HAT) activity. Therewith, a

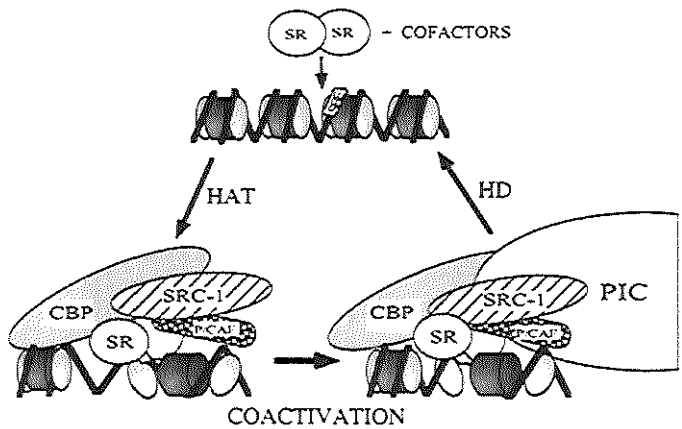


Figure 2.2.4: Two step model of steroid receptor-induced gene transcription. Liganded steroid receptors (R), that are able to bind a steroid response element in chromatin repressed DNA, can recruit cofactors such as steroid receptor coactivator 1 (SCR-1), coactivator CREB (cAMP- response element-binding protein)-binding protein (CBP) and p300/CBP-associated factor (P/CAF). These cofactors possess intrinsic histone acetyltransferase (HAT) activity that can loosen nucleosomal structure by targeted histone acetylation. Coactivators, such as SRC-1 and CBP, can then initiate stable assembly of the preinitiation complex (PIC) by "coactivation" that results in enhanced rates of transcription initiation by RNA polymerase II. The effects of targeted histone acetylation may be reversed by histone deacetylation (HD). Furthermore, histone deacetylation may be responsible for turning off activated transcription once the PIC and/or steroid receptor complex becomes unstable (from Jenster et al., 1997).

third model arises, in which co-activator induced chromatin remodeling is recognized as an important feature in the steroid receptor transactivation of target genes. Jenster et al (Jenster et al., 1997) have integrated these different models into a "two-step model of steroid receptor transactivation in vivo", which is shown in figure 2.2.4.

Non-genomic regulation of hPR mediated transcription activation

Progesterone receptor has also been suggested to be involved in indirect regulation of gene transcription. Migliaccio et al (Migliaccio et al., 1998) suggest a cross-talk between hPR and hER in stimulation of the c-Src/$p21^{ras}$/MAP kinase signaling pathway. hPR could only stimulate this pathway when associated with the hER. Direct interaction of the hPR with the c-Src/$p21^{ras}$/MAP kinase pathway could not be observed. Subsequently, the first164 amino acid residues of the hPR, which are unique for the hPRB isoform, were identified as the domain responsible for association with the hER. Progesterone can also influence gene expression via binding to transmembrane receptors. Putative transmembrane progesterone receptors have been identified in humans (Gerdes et al., 1998, Bernauer et al., 2001) and progesterone has been shown to bind to the transmembrane uterine oxytocin receptor directly (Grazzini et al., 1998). Liganded hPR has also been suggested to interfere with the activity of other transcription factors, such as ER, by squelching. Squelching means that hPR competes with other transcription factors for binding of common coactivators, which then will be less available for the other transcription factors to activate their target genes (Meyer et al., 1989).

Transcription regulation differences between hPRA and hPRB

As mentioned earlier, apart from the first 164 NH_2-terminal amino acids, the two hPR isoforms (hPRA and hPRB) are structurally identical. However, hPRA and hPRB are not functionally identical. In transfection experiments, it has been observed that their relative efficiency to activate target genes vary according to the promoter and cell context used (Tora et al., 1988; Meyer et al., 1989). HPRB has been thought to be more transcriptionally active than hPRA. Moreover, hPRA has been shown to act as a strong trans-dominant repressor of hPRB (and other steroid hormone receptors) mediated transcription in specific promoter and cell contexts (Vegeto et al., 1993). However, recently Gao et al. (Gao et al., 2000) observed that in endometrial stroma cells hPRA acted as a stronger transactivator than hPRB for the IGFBP-1 (insuline-like

growth factor binding protein-1) promoter. Moreover, in transgenic mice, overexpression of PRA resulted in a significant different phenotype (Shyamala et al., 1998) compared to wild-type, and in PRA knockout (PRAKO) mice, Mulac-Jericevic demonstrated PRB and PRA specific gene regulation (Mulac-Jericevic et al., 2000).

The mechanisms responsible for the distinct transcriptional properties of the two PR isoforms are largely unknown. However, recently several models have been proposed to be involved in these functional differences between hPRA and hPRB.

1) *Differences in phosphorylation sites between hPRA and hPRB* — Reversible protein phosphorylation is one of the most important mechanisms of regulating intra-cellular processes. Through phosphorylation and dephosphorylation, protein functions can be switched on or off. In the hPR several serine residues, all located in the NH_2-terminal domain, have been identified to be phosphorylated (Clemm et al., 2000). Phosphorylation of these sites is regulated by different signal transduction pathways (Zhang et al., 1994; Zhang et al., 1997). Recently, Clemm et al. (Clemm et al., 2000) showed that one of the serine sites (Ser 294) exhibited a stronger hormone-dependent phosphorylation in hPRB compared to hPRA. This Ser 294 is located within the inhibitory domain (ID). Subsequently, Clemm et al have suggested that a distinct conformation of the NH2 terminus of hPRA inhibits phosphorylation of this site and results in differential functional properties of hPRA and hPRB.
2) *Differences in AFs (Activation Funtions) between hPRA and hPRB* — Hovland et al. (Hovland et al., 1998) have shown that the inhibitory domain (ID), located in the NH_2-terminus, inhibits both AF-1 and AF-2 but not AF-3. The AF-3 is located within the first 164 NH2-terminal amino acid residues, and is unique for hPRB. Subsequently, Hovland et al. (Hovland et al., 1998) hypothesize that hPRA is transcriptionally inactive because, in the absence of AF-3, the ID prevents AF-1 and AF-2 from activating transcription. Moreover, in hPRB, AF-3 is proposed to overrule the inhibitory function of ID, thereby allowing hPRB to activate transcription (Giangrande et al., 1997; Hovland et al., 1998).
3) *Differences in interaction with coactivators* — Recently, it has been shown that the amino termini of hPRB and hPRA interact differently with the carboxyl terminus of the LBD (Tetel et al., 1999). The NH_2 terminus of

hPRB, but not that of hPRA, interacts efficiently with its LBD in an agonist-dependent manner. Differential interaction between carboxyl and amino termini can regulate the ability of the hPR to interact with cofactors in a productive manner (Giangrande et al., 2000). Subsequently, Giangrande et al. (Giangrande et al., 2000) has shown that, upon agonist binding, hPRA, unlike hPRB, is unable to efficiently recruit the transcriptional coactivators GRIP1 and SRC-1. This may contribute to the transcriptional inactivity of hPRA in specific cell and/or promoter context. Moreover, they observed that hPRA does have a higher affinity for the corepressor SMRT compared to hPRB, which could account for the transdominant repressor function of hPRA in specific cell/promoter context (as observed by Vegeto et al.,(Vegeto et al., 1993).

2.2.4 Progesterone target genes

So far, only a small group of progesterone target genes have been identified. The hPR gene itself and the ER gene are the most well-known progesterone-regulated genes (Read et al., 1988). The genes encoding fatty acid synthetase, metallothionein-IIa, alkaline phosphatase, monocyte chemotactic proteins, vascular endothelial growth factor, the proteases: ADAMTS-1 and cathepsin, Sox4, epidermal growth factor and its receptor are other genes reported to be target genes for progesterone (Chalbos et al., 1986; Slater et al., 1988; Asselin et al., 2001; Classen-Linke et al., 2000; Robker et al., 2000; Graham et al., 1999; Musgrove et al., 1991). Ten other progesterone target genes have been identified in the breast cancer cell line T47D by Kester et al.,(Kester et al., 1997), using a differential display technique. They found TSC-22, CD-9, CD-59, desmoplakin FKBP51, Na/K-ATPaseα1 and three ESTs to be up-regulated by progestin in these breast cancer cells and annexin-VI down-regulated. In the endometrial carcinoma cells ECC-1, only CD-59, desmoplakin and FKBP51 were induced by progestin, indicating that the progestin-responsiveness of some of these progesterone target genes might be cell type-specific (Kester, 1999). In mice, the expression of genes encoding calcitonin, histidine decarboxylase, amphiregulin, Hoxa10 and lactoferrin was found to be progesterone responsive (Zhu et al., 1998; Paria et al., 1998; Das et al., 1995; Ma et al., 1998; McMaster et al., 1992). As hPRA and hPRB exhibit distinct functions, the existence of hPRA and hPRB specific target genes has been suggested. Recently, this hypothesis was supported by the observation in PRA

knockout mice (Mulac-Jericevic et al., 2000) that the progesterone responsiveness of genes encoding calcitonin and amphiregulin is lost in these mice compared to wild-type mice, indicating that the regulation of these genes might be PRA specific.

2.3 Genes and endometrial cancer

2.3.1 Genetic models for carcinogenesis

Most cancers are thought to originate from a single cell, which has escaped normal cellular growth control mechanisms. This escape results from functional changes in expression levels of genes, which are key role players in controlling cell growth, DNA repair, or programmed cell death. In general, three groups of genes are thought to be involved: tumor-suppressor genes, (proto-)oncogenes and DNA-repair genes. Tumor-suppressor genes encode proteins, which inhibit proliferation. Inactivation of tumor-suppressor genes can lead to uncontrolled cell growth and contribute to the development of cancer. Proto-oncogenes are genes, which encode proteins that stimulate growth factor signaling pathways in cells. Proto-oncogenes will become oncogenes when the encoded protein is either overexpressed or hyperactive. DNA-repair genes are involved in mending replication errors and maintaining genetic stability. Inactivation of these genes will render the genome vulnerable to mutational events and will lead to genetic instability. Genetic alterations, such as chromosomal rearrangements, deletions and point mutations, can lead to a complete loss of the gene transcript. However, deletions or point mutations in the coding sequences can also result in a hyperactive or inactive protein without changes in the expression levels of the corresponding mRNA. Moreover, chromosomal rearrangement can position a gene near an enhancer sequence, causing changes in the expression level of the gene product or creating either a hyperactive or an inactive fusion product.

A number of models have been developed to describe the genetic mechanisms involved in carcinogenesis. Based on a hereditary form of retinoblastoma, Knudson (Knudson, 1971) developed a "two-hit" scenario in which hereditary forms of cancer develop according to a fairly simple scheme (Figure 2.3.1). In this scheme the "first-hit" in the development of hereditary cancer

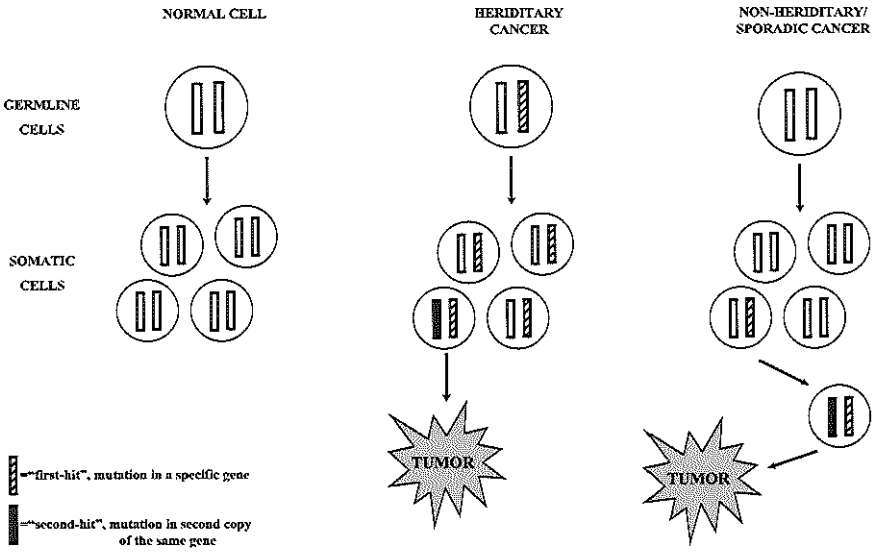


Figure 2.3.1: Knudson's "two-hit" scenario for the development of cancer. The first "hit" would be a mutation in a specific cancer gene, in the germline in a hereditary case, in a somatic cell in a non-hereditary case. The second "hit" in both groups would be a somatic mutation in, or loss of, the second copy of the same gene (adapted from Knudson (Knudson, 1985), (Knudson, 1993)).

counts for an already mutated allele of a specific cancer gene in the germline cell. Cancer arises only after a "second-hit" has occurred. This "second-hit" stands for a mutation in the other allele of the same gene in the somatic cell. Knudson's "two-hit" scenario can also be extended to the carcinogenesis of sporadic, non-hereditary cancers; the "first-hit" then occurs in a somatic cell, which will result in a sporadic, non-hereditary cancer; again only after a second-hit has occurred. However, for the majority of cancers the genetic mechanisms involved in the carcinogenesis are much more complex. Although inactivation of tumor-suppressor genes does occur in accordance with Knudson's two-hit-model, in most cancers an accumulation of genetic events is needed before a tumor develops. Already in 1958 Foulds postulated such a multi-step model for carcinogenesis. Fearon and Vogelstein (Fearon & Vogelstein, 1990) have given an elegant example of a multi-step scenario using colorectal cancer as a model. In this model, accumulation of mutations in both proto-oncogenes and tumor-suppressor genes direct the progression

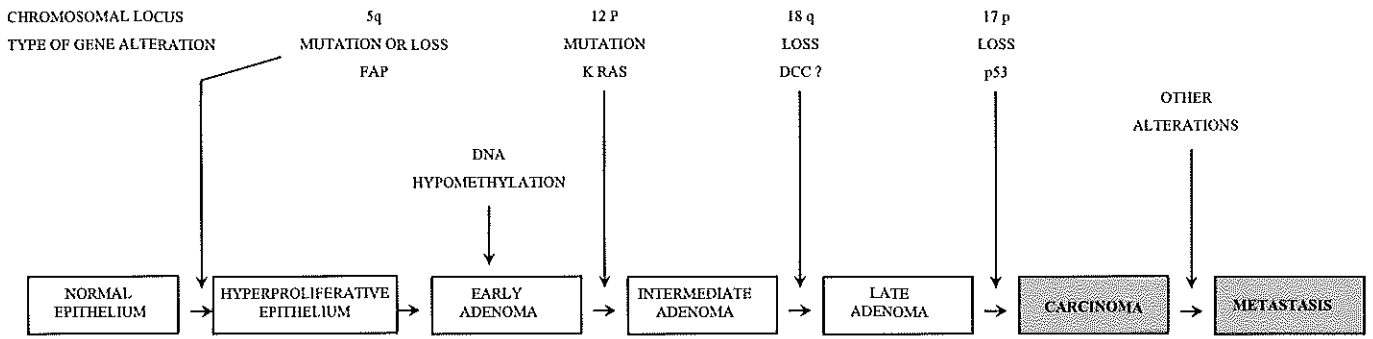


Figure 2.3.2: A genetic model for colorectal tumorigenesis. Tumorigenesis proceeds through a series of genetic alterations involving oncogenes and tumor-suppressor genes. The three stages of adenomas represent tumors of increasing size, dysplasia, and villous content. In patients with familial adenomatous polyposis (FAP), a mutation on chromosome 5q is inherited. In tumors arising in patients without polyposis, the same region may also be lost and/or mutated at a relatively early stage of tumorigenesis. Hypomethylation is present in very small adenomas in patients with or without polyposis, and this alteration may lead to aneuploidy, resulting in the loss of suppressor gene alleles. K-ras gene mutations appear to occur in one cell of a preexisting small adenoma and, through clonal expansion, produce a larger and more dysplastic tumor. The chromomes most frequently deleted include 5q, 17p and 18q: the putative target of the loss event (i.e. the tumorsuppressor gene) on each chromosome is indicated as well as the relative timing of the loss event. Allelic deletions of chromosome 17p and 18q usually occur at a later stage of tumorigenesis than do deletions of chromosome 5q or K-ras gene mutations. However, the order of these changes is not invariant, and accumulation of these changes, rather than their order with respect to one another, seems most important. Tumors continue to progress once carcinomas have formed, and the accumulated loss of suppressor genes on additional chromosomes correlates with the ability of the carcinomas to metastasize and cause death (adapted from Fearon and Vogelstein (Fearon & Vogelstein, 1990)).

of normal colorectal epithelial tissue via pre-malignant stages into a colorectal carcinoma (Figure 2.3.2).

During the process of DNA replication, many accidental mutations occur in a cell. Normally the cellular mismatch repair system will detect and mend these lesions. Loss of DNA repair activity will lead to a cell that becomes highly at risk for genetic alterations. Loeb et al described these cells as cells with a *mutator phenotype* (Loeb, 1991). Many events can contribute to genetic changes. Mutations can occur as spontaneously arising "errors" during the process of mitosis, but can also be caused by environmental factors such as chemical or physical agents. Moreover, viruses can insert DNA into the genome, and subsequently act as an oncogene or as an enhancer to nearby proto-oncogenes. Not only genetic but also epigenetic events such as imprinting, aberrant methylation, formation of DNA adducts and post-translational modifications can play a role in tumorigenesis. Also cellular or environmental factors that stimulate proliferation of an initiated cell will increase the probability of additional mutational or epigenetic changes and therewith contribute to the process of carcinogenesis.

All these observations point to a rather complex multi-step carcinogenesis model in which genetic, epigenetic and environmental factors are involved.

2.3.2 Endometrial cancer

The molecular processes involved in tumorigenesis of endometrial cancer are largely unknown. On basis of histological and clinical characteristics two pathways have been suggested in endometrial carcinogenesis (Deligdisch & Holinka, 1987, Sherman et al., 1995b, Nogales, 1996, Esteller et al., 1999, Sherman et al., 1995a, Zheng et al., 1996, Kurman Blaustein's Pathology of the Female Genital Tract, Springer-Verlag 1994): An estrogen-driven pathway leading to atypical hyperplasia of the endometrium which slowly progresses into an endometrioid carcinoma (Kurman et al., 1985, Lindahl & Willen, 1991) and an estrogen-unrelated pathway leading to the aggressive papillary serous or clear cell carcinomas. Papillary serous carcinomas are found to arise in atrophic endometrium and are associated with a high grade precursor lesion: endometrial intraepithelial carcinoma (EIC) (Ambros et al., 1995). The differences in estrogen-sensitivity and the different cellular precursor lesions suggest a different genetic pathway for each of the two subtypes. How-

ever, a genetic background supporting the division of endometrial cancers into these two groups, or other groups, has yet to be established.

2.3.3 Genes in endometrial cancers

So far, little attention has been paid to heredity in the etiology of endometrial cancer. Endometrial cancer is known to be the most common extra-colonic cancer in HNPCC (hereditary nonpolyposis colorectal carcinoma) (Mecklin & Jarvinen, 1991). Women carrying mutations in the DNA mismatch repair genes known to be associated with HNPCC (e.g. hMSH2, hMLH1, hPMS2 and hMSH6 genes) are found to have a 22-43% lifetime risk of developing endometrial cancer, which is significant higher than the lifetime risk in the general Dutch or USA female population (1.7-3.3%) (Watson & Lynch, 1993, Aarnio et al., 1995, Dunlop et al., 1997, O Visser et al., Incidence of Cancer in the Netherlands 1995, Association of Comprehensive Cancer Centers 1998). No other germline mutations have been identified in endometrial cancer yet.

Mutations and expression levels of several cancer related genes have been studied in sporadic endometrial cancers. The following paragraph and Table 2.3.1 give an overview of the most frequently studied genes in endometrial cancer.

DNA-repair genes

DNA-repair or mismatch repair genes are involved in mending DNA replication errors and maintaining genetic stability. In normal cells errors from DNA replication which have escaped the proofreading activity of DNA polymerase will be repaired by the DNA mismatch repair system (MMR). Defects in this MMR system can result in a RER+ (**R**eplication **Er**rors) or mutator phenotype of the effected cell (Loeb, 1991). Randomly throughout the genome short tandem repeat sequences occur. These repeated sequences are called microsatellites. Due to their repeating nature, these microsatellites are particularly prone to errors during replication. RER+ cells can be recognized by instability of their microsatellites (Ionov et al., 1993). Microsatellite instability (MSI) has first been detected in hereditary nonpolyposis colorectal carcinomas (HNPCC) (Aaltonen et al., 1993) and subsequently in many other tumors. In endometrial cancer MSI has been observed in 15-34% of the tumors, suggesting a role for DNA-repair genes in the carcinogenesis of

Table 2.3.1: Genes in endometrial cancer.

gene	type of abnormality	references	number cases/ type+stage studied	percentage affected	independent prognostic factor?	correlation with...
DNA-repair						
hMSH2,	mutation	Katabuchi, 1995	12 MSI-positive endometrioid	8%	-	-
	loss expression	Katabuchi, 1995	12 MSI-positive endometrioid	18%	-	-
		Parc, 2000	21 MSI-positive adenocarc	19%	-	-
	methylation promoter	Salvesen, 2000	138 endometrial carc	1%	-	-
hPMS2	mutation	Basil, 1999	40 MSI-positive adenocarc	0%	-	-
hMLHI	methylation promoter	Gurin, 1999	14 MSI-positive endometrioid	71%	-	-
		Salvesen, 2000	138 endometrial carc	23%	no	-
		Simpkins, 1999	53 MSI-positive adenocarc	77%	-	-
	loss expression	Parc, 2000	21 MSI-positive adenocarc	57%	-	-
		Salvesen, 2000	138 endometrial carc	14%	no	MSI pos., p53, methylation
MSI						
MSI		Sakamoto, 1998	72 (non-)endometrioid	15%	-	-
		Risinger, 1993	36 (non-)endometrioid	17%	-	early stage
		Burks, 1994	30 (non-)endometrioid	23%	-	hist.type
		Duggan, 1994	45 (non-)endometrioid	20%	-	hist.type
		Parc, 2000	62 endometrial carc	34%	-	high stage
		Tibiletti, 1999	51(non-)endometrioid	20%	-	hist.type, not stage/ grade
		Lim, 1996	28 endometrial carc	32%	-	-
		Katabuchi, 1995	65 (non-)endometrioid	18%	-	hist.type
		Gurin, 1999	57 endometrial carc	25%	-	-

Tumor-suppressor						
p53	mutation or overexpression	Kohler, 1992	107 endometrial carc	21%	multiv. yes	high stage
		Enomoto, 1993	40 endometrial carc (+13 hyperpl)	23% (8%)	-	high grade
		Lukes, 1994	100 (non-)endometrioid	27%	univ. yes	-
		Reinartz, 1994	128 (non-)endometrioid	29%	univ. yes	hist. type, stage, grade
		Ito, 1994	221 endometrioid	21%	multiv. yes	high stage
		Kohler, 1996	179 stage III/IV endometrial carc	35%	multiv. yes	black race
		Zheng, 1996	21 endometrioid / 21 PSC	29% / 71%	-	stage EC / not stage PSC
		Kohlberger, 1996	92 stage I endometrial carc	9%	multiv. yes	not hist. type, not stage
		Powell, 1999	179 stage I endometrial carc	6%	multiv. yes	-
		Bell, 1997	48 stage I endometrial carc	-	no	-
		Bancher-Todesca, 1998	23 papillary serous (PSC)	48%	univ. yes	high stage
		Coppola, 1998	50 endometrial carc	22%	no	not stage
		Ioffe, 1998	18 stage I EC/ 36 hyperpl/ 10 normal	50% / 0 / 0	-	cell turnover indices
		Backe, 1997	202 endometrial carc	31%	univ. yes	grade
		Geisler, 1999	137 (non-)endometrioid	(94%-) 57%	multiv. yes	high stage
		Lim, 1999	42 stage I endometrioid	24%	multiv. yes	-
		Silverman, 2000	134 curettage material	40%	multiv. yes	hist.type, stage, not grade
PTEN /	mutation	Tashiro, 1997	32 (non-)endometrioid	(0%-)50%	-	MSI-positive, hist.type
MMAC1		Risinger, 1997	70 endometrial carc	34%	-	-
		Kong, 1997	38 endometrial carc	55%	-	MSI-positive
DCC	mutation or loss expression	Saegusa, 1999	37 endometrial carc	35%	-	not stage
		Enomoto, 1995	8 endometrial carc	50%	-	-
		Gima, 1994	28 endometrial carc	50%	no	not stage
IGF-IIR	mutation	Catasus, 2000	24 MSI-positive endometrioid	12%	-	-
		Ouyang, 1997	26 MSI-positive endometrial carc	15%	-	early stage, low grade
IGF-IR		Maiorano, 1999	30 endometrial carc	0%	-	-

Table 2.3.1: Genes in endometrial cancer - *continued*

gene	type of abnormality	references	number cases/ type+stage studied	percentage affected	independent prognostic factor?	correlation with...
Tumor-suppressor						
TGFbeta-R2	mutation or loss expression	Risinger, 1997	70 endometrial carc	0%	-	-
		Gold, 1994	-	0%	-	-
		Myeroff, 1995	MSI-positive endometrial carc	17%	-	-
		Catasus, 2000	24 MSI-positive endometrioid	0%	-	-
Rb	mutation or loss expression	Niemann, 1997	70 endometrial carc	6%	-	high stage
		Milde-Langosch, 1999	36 (non-)endometrioid	3%	-	not stage, not grade
		Semczuk, 2000	62 (non-)endometrioid	5%	-	high stage
		Yaginuma, 1996	20 (non-)endometrioid	10%	-	-
Oncogene						
K-ras	mutation	Lax, 2000	58 EC / 42 PSC	26% / 2%	-	hist.type, not grade
		Fujimoto, 1993	45 endometrial carc	22%	-	not hist. type, not stage
		Enomoto, 1991/1993	52 endometrial carc	29%	-	-
		Sasaki, 1993	84 endometrial carc	18%	-	high in Japanese race
		Duggan, 1994	60 endometrial carc	12%	-	-
		Mutter, 1999	56 endometrial carc	15%	-	also in hyperplasia
		Jones, 1997	32 (non-)endometrioid	19%	no	-
		Esteller, 1997	55 endometrial carc	15%	no	not hist. type, not stage
		Semczuk, 1997	13 endometrial carc	15%	-	high stage
		Semczuk, 1998	57 endometrial carc	14%	no	hist.type (clear cell)
		Niederacher, 1999	112 endometrial carc	12%	no	not stage, not grade
c-fms	mutation or overexpression	Leiserowitz, 1993	32 (non-)endometrioid	-	-	high grade
		Smith, 1995	71 endometrial carc	50%	-	-
		Baiocchi, 1991	9 endometrial carc	67%	-	-

c-myc	mutation or overexpression	Niederacher, 1999	112 endometrial carc	3%	no	not stage, not grade
		Monk, 1994	37 endometrial carc	11%	-	high grade
c-*erb*B-2 / HER-2/ *neu*	mutation or overexpression	Wang, 1993	34 (non-)endometrioid	15%	no	not stage, not grade
		Hetzel, 1992	247 endometrial carc	15%	multiv. yes	high stage
		Berchuck, 1991	95 endometrial carc	10%	-	increased mortality
		Reinartz, 1994	128 (non-)endometrioid	2%	no	hist.type
		Silverman, 2000	134 curettage material	12%	no	early stage, hist.type
		Lukes, 1994	100 (non-)endometrioid	12%	univ. yes	-
		Rolitsky, 1999	72 endometrial carc	21%	multiv. yes	higher grade, hist.type
	amplification	Riben, 1997	63 stage I endometrial carc	38%	multiv. yes	-
		Rolitsky, 1999	72 endometrial carc	17%	multiv. yes	higher grade, hist.type
		Saffari, 1995	92 endometrial carc	21%	multiv. yes	-
bcl-2	mutation or overexpression	Zheng, 1996	42 (non-)endometrioid	-	-	hist.type
		Ioffe, 1998	18 stage I endometrial carc	38%	-	high cell turnover
	expressed	Coppola, 1998	50 endometrial carc	85%	no	high stage
		Morsi, 2000	26 endometrial carc	-	-	low grade
		Geisler, 1998	91 EC / 29 non-endometrioid	44% / 23%	multiv. yes	early stage, low grade

Several oncogenes, tumor-suppressor genes and DNA-repair genes have been studied with regard to their possible involvement in tumorigenesis of endometrial cancer. This table gives an overview of the most frequently genes studied. Four groups (class) have been made: DNA-repair genes, microsatellite instability, tumor-suppressor genes and oncogenes. Named are the genes studied (gene), the type of genetic alteration found in the gene (type abnormality), the references (references), the number of patients studied and the histological type and stage of the tumor studied (number of cases/type and stage studied), the percentage of cases in which the genetic alteration was found (percentage affected), whether the gene was found to be an independent prognostic factor for disease free survival, either by multivariant or univariant analysis (independent prognostic factor) and whether the found gene alteration was correlated with any other characteristic of the tumor (correlated with...). – = no data were available. Carc = carcinoma. EC = endometrioid carcinoma. PSC =papillary serous carcinoma. Hyperpl = hyperplasia. MI = MSI = microsatellite instability. Multiv. = multivariate. Univ. = univariate. Stage and grade refer to the FIGO staging system and histological grading.

endometrial cancer. Inactivation of the DNA repair genes can result from genetic alterations in these genes. In HNPCC, germline mutations in several DNA-repair genes (hMSH2, hMLH1, hPMS2 and hMSH6) have been identified (Fishel et al., 1993, Bronner et al., 1994, Papadopoulos et al., 1994, Malkhosyan et al., 1996). However, endometrial tumors rarely show mutations in these DNA-repair genes (Kowalski et al., 1997, Katabuchi et al., 1995, Kobayashi et al., 1996, Lim et al., 1996). Simpkins et al. (Simpkins et al., 1999) have suggested an alternative mechanism for inactivation of DNA-repair genes. They found methylation of the hMLH1 promoter in 77% of MSI positive tumors, and also loss of hMLH1 protein expression but no mutations in the genes. Subsequently they suggested that not genetic but epigenetic alterations of DNA-repair genes account for the RER+ phenotype in a large group of endometrial cancers. Salvesen et al. (Salvesen et al., 2000) confirmed Simpkins' observations. Studying 138 endometrial carcinomas, they found methylation of the hMLH1 promoter in 69% of the tumors with high MSI compared to 10% in MSI-negative tumors. Loss of hMLH1 nuclear staining was seen in 93% of the methylated MSI-positive tumors. All unmethylated tumors showed normal expression of hMLH1. Salvesen et al. (Salvesen et al., 2000) conclude that hMHL1 methylation is significantly correlated with loss of hMLH1 expression, lack of p53 overexpression, and with microsatellite instability (MSI).

In endometrial cancers, MSI is found to be restricted to tumors of the endometrioid type. No association was found between MSI and tumor stage or grade. These observations could indicate that MSI is an early event associated with the development of endometrioid endometrial cancers and could be caused by hyper-methylation of DNA-repair gene promoters.

Other genes, containing short repeating sequences, are also prone to genetic alterations in a cell with a mutator phenotype. *TGF-beta receptor II / IGF-II receptor* are tumor-suppressor genes, and known to carry short repeating sequences. Several groups have studied endometrial tumors for the presence of mutations in these genes (Table 2.3.1). However, mutations in these genes appear to be a rare event in endometrial cancers. Even in endometrial tumors carrying microsatellite instability, mutations are only observed in 0-17% of the tumors.

Tumor-suppressor genes

Tumor-suppressor genes are genes normally encoding proteins involved in

inhibition of cell proliferation. Complete or partial deletion, mutation or chromosomal rearrangement can cause functional damage to the genes, resulting in either complete loss of the gene transcript or production of an aberrant protein. p53, PTEN and DCC are the most frequently found mutated tumor-suppressor genes in endometrial cancers.

- *P53* – Mutations of the tumor-suppressor gene p53 belong to the group of ubiquitous genetic alterations in human tumors (Hollstein et al., 1991). Wildtype p53 functions as a G1-S checkpoint. p53 induces a G1 arrest, creating extra time for DNA-repair mechanisms. If DNA-repair fails, p53 may initiate cell death via apoptosis (Lane, 1992). Mutations in the p53 gene can result in a protein with increased stability. Subsequently, due to the longer half-life of the mutant protein, the functional inactive p53 protein becomes overexpressed in the tumor cell (Finlay et al., 1988). Total loss of p53 protein expression is a relatively uncommon event, but can result from non-sense mutations or complete deletion of p53 gene (Hollstein et al., 1991). Several groups have studied the expression level of p53 protein in endometrial cancers using immunohistochemical staining. A significant difference has been found between the expression of p53 in the two histological endometrial cancer subtypes: p53 is less frequently found to be overexpressed in endometrioid carcinomas: 17-57 %, as compared to the more aggressive papillary serous endometrial carcinomas: 47-94 % (Kohler et al., 1992, Geisler et al., 1999, Ito et al., 1994, Lax et al., 2000, Bancher-Todesca et al., 1998). In endometrioid tumors, the percentage of tumors with p53 overexpression is significantly lower in stage I/II tumors as compared to stage III/IV tumors (Kohler et al., 1992, Geisler et al., 1999, Ito et al., 1994, Lax et al., 2000). However, in papillary serous carcinomas, p53 overexpression seems to represent an early event in carcinogenesis, as the same percentage of p53 overexpression is found in all FIGO stages (Geisler et al., 1999, Bancher-Todesca et al., 1998, Zheng et al., 1996). These observations suggest that p53 expression could function as a prognostic marker for disease-free survival. A majority of reports show p53 overexpression to be an independent prognostic marker in multivariate analysis, confirming the prognostic value of p53 (Geisler et al., 1999, Silverman et al., 2000, Kohler et al., 1992, Kohlberger et al., 1996, Ito et al., 1994, Powell et al., 1999 and Lim et al., 1999). However, Lukes et al. (Lukes et al., 1994, Blacke et al., '97 and Reinartz

et al., 1994), whilst finding a correlation using univariant analysis, but multivariate analysis failed to show a relation in their studies. Contradictory results were also observed by Bell et al. (Bell et al., 1997) who studied 349 stage I endometrial carcinomas and found no relationship between p53 expression and disease recurrence. Bancher-Todesca et al. (Bancher-Todesca et al., 1998) found no significant association between survival and p53 expression in papillary serous carcinoma. These last two observations could be explained as a result of a correlation between p53 expression and higher stage and aggressive histological subtypes. However, the studies of Kohlberger et al. (Kohlberger et al., 1996) and Lim et al. (Lim et al., 1999) challenge such a correlation, as they did find, in stage 1 endometrial tumors, p53 to be a multivariate independent prognostic value for disease free survival. All studies used immunohistochemical staining to evaluate p53 expression in the tumor samples. However, methods to quantify the staining results, cut off levels for defining overexpression and the antibodies used, differ between the different studies. These differences in methodology could influence the obtained results. Moreover, in a recent study by Lax et al. (Lax et al., 2000) it was found that in 15% of the p53 mutations no immunoreactivity for p53 protein was detectable, indicating that immunohistochemical staining studies underestimate p53 mutation frequency.

Different roles of p53 in the carcinogenesis of the two histological subtypes have been proposed. Overexpression of p53 is rare in premalignant hyperplastic endometrium: 0-8% (Enomoto et al., 1993, Kohler et al., 1992, Ioffe et al., 1998), and in stage 1 endometrioid carcinomas: 0-21% (Kohler et al., 1992, Kohlberger et al., 1996, Lim et al., 1999). Therefore, p53 mutations appear to be late events in the development of endometrioid carcinoma. In contrast, p53 overexpression appears to represent an early event in serous carcinomas, as the precursor lesion EIC (endometrial intraepithelial carcinoma) is always associated with p53 overexpression in p53 positive serous carcinomas (Sherman et al., 1995a). These observations suggest that p53 mutation is associated with an aggressive, invasive type of endometrial carcinoma cells and may serve as a prognostic marker for disease-free survival in endometrial cancers.

- *PTEN* — An other common genetic alteration found in human tumors is mutation of the tumor-suppressor gene PTEN/MMAC1. Germline muta-

tions of the tumor-suppressor gene PTEN are found in Cowden disease, an autosomal dominant cancer predisposition syndrome characterized by multiple hamartomas in various organs (breast, skin, thyroid) (Liaw et al., 1997) and in the Bannayan-Zonana syndrome characterized by macrocephaly lipomas, intestinal hamartomatous polyps vascular malformations and skin disorders (Marsh et al., 1997). PTEN has an intrinsic phosphatase activity and functions as a tumor suppressor by counteracting the oncogenic effects of tyrosine kinases (Furnari et al., 1997). PTEN has been shown to inhibit cell migration. Therefore, loss of PTEN function may stimulate tumor progression and metastasis (Tamura et al., 1998). PTEN mutations were detected in 34%-55% (Tashiro et al., 1997), (Risinger et al., 1997, Kong et al., 1997) of endometrial carcinomas. Mutations seem to be confined to the endometrioid histological subtype (Tashiro et al., 1997, Risinger et al., 1997). PTEN is more often found mutated in MSI-positive endometrial carcinomas than in MSI-negative tumors, and does not appear to be stage related (Tashiro et al., 1997, Kong et al., 1997, Risinger et al., 1997). Therefore, it has been hypothesized that PTEN mutations occur as an early event in carcinogenesis of endometrioid endometrial carcinomas that already have acquired a mutator phenotype. In the development of papillary serous endometrial carcinomas, PTEN mutations do not seem to play a significant role.

- *DCC* — The tumor-suppressor gene DCC (**D**eleted in **C**olorectal **C**arcinoma) was first found mutated in colorectal cancers. The DCC protein plays a role both in cell growth and differentiation (Hedrick et al., 1994). In colorectal cancers, DCC mutations are thought to be a late event in carcinogenesis, as low DCC protein expression is found to be associated with a increased risk of developing metastases and with lower five-years survival rates (Itoh et al., 1993, Saito et al., 1999, Schmitt et al., 1998, Gotley et al., 1996). Similar observations have been made by three different research groups investigating DCC expression in endometrial carcinomas (Saegusa et al., 1999, Enomoto et al., 1995, Gima et al., 1994). They reported DCC mutations and loss of protein expression in 35-50% of the endometrial carcinomas. No association has been found with either FIGO stage or disease outcome. However, in all three studies only a small number of endometrial carcinoma samples were investigated.
- *Rb* — The retinoblastoma gene (Rb) was the first described tumor-suppressor gene and was originally found to be the gene responsible for the

hereditary retinoblastoma syndrome (Lee et al., 1987, Friend et al., 1986, Horowitz et al., 1990). Subsequently, mutations in the Rb gene have been detected in a number of other tumors, such as breast, lung, prostate and bladder (Ishikawa et al., 1991, Yokota et al., 1988, Lee et al., 1987, Bookstein et al., 1990a; Bookstein et al., 1990b). However, in endometrial cancer alterations in the Rb gene seem to be a rare event. Yaginuma et al. (Yaginuma et al., 1996) found LOH at the Rb locus in 10% of endometrial carcinomas. Using immunohistochemical staining (Niemann et al., 1997, Semczuk et al., 2000, Milde-Langosch et al., 1999), loss of Rb protein expression has been found in only 3%-6% of the cases.

Oncogenes

Proto-oncogenes are genes that normally encode proteins that stimulate the growth signaling pathways in cells. They can become tumorigenic either when their protein is overexpressed or hyperactive. After this functional change their names change accordingly from proto-oncogenes to oncogenes. Several genetic events may cause this transition. Deletions or point mutations in the coding sequence can result in a hyperactive protein, even without changing the expression level of the gene. Chromosomal rearrangements can position a gene near an enhancer, increasing the expression level of the gene or creating a hyperactive fusion product. Moreover, viruses can integrate DNA into the genome, and subsequently act as an oncogene or as an enhancer to nearby proto-oncogenes.

- *K-ras* — The proto-oncogene K-ras is involved in controlling cell proliferation. The activated Ras protein triggers activation of the MAP kinase pathway and subsequently stimulates cell proliferation via activation of several growth factors (Hill et al., 1993). In endometrial carcinomas, codon 12 of the K-ras gene is the most frequent site of mutations, causing a constitutive active product. Mutations in codon 12 have been found in 12-29% of endometrial tumors. Moreover, race-related differences in mutation rate have been reported, with higher frequency of mutations in Japan (22-29%) as compared to USA (12-19%) (Sasaki et al., 1993, Caduff et al., 1995). Activation of K-ras has been suggested to represent an early event in the development of endometrial cancer, as mutations have also been found in endometrial hyperplasia. K-ras has been shown to cross-talk with the estradiol signaling pathway, as activation of K-ras has been

reported to enhance estrogen-induced and anti-estrogen-induced transcriptional activity of the estrogen receptor activation function (Bunone et al., 1996, Kato et al., 1995). This suggests that mutational activation of the ras-MAPK cascade may be involved in the development of hormone independency in endometrial cancer (Niederacher et al., 1999). However, so far no correlation has been found between K-ras mutations and specific histological characteristics or prognosis of endometrial cancers.

- *C-myc* – Proto-oncogene c-myc is an early response gene and essential in controlling cell proliferation. Mutations in c-myc appear to be a relatively rare event in endometrial carcinomas: 3-11% (Niederacher et al., 1999, Monk et al., 1994).
- *C-fms* – Proto-oncogene c-fms encodes a transmembrane tyrosine kinase receptor for the colony-stimulating factor 1 (CSF-1). CSF-1 is a growth factor, regulating growth and differentiation of mononuclear phagocytes. In the human endometrial adenocarcinoma cell line Ishikawa, CSF was found to inhibit growth and induce differentiation (Kimura et al., 1991). Overexpression of the CSF receptor c-fms is found in 50-67% of the endometrial carcinomas and is correlated with high-grade tumors (Leiserowitz et al., 1993, Smith et al., 1995, Baiocchi et al., 1991). No correlation has been found between c-fms expression and a specific histological subtype of the endometrial tumors.
- *C-erb B (HER-2/neu)* – The proto-oncogene erb-B encodes an EGF (epidermal growth factor) receptor. When EGF binds to the receptor, the intracellular tyrosine kinase domain becomes activated and starts a signaling pathway leading to cell proliferation. The mutated erbB (c-erbB) encodes a truncated form of the EGF receptor that is constitutively active. Berchuck et al. (Berchuck et al., 1991), Hetzel et al. (Hetzel et al., 1992), Lukes et al. (Lukes et al., 1994) and Rolitsky et al. (Rolitsky et al., 1999) found c-erbB overexpression to be an independent prognostic factor for disease free survival in endometrial cancers. In these 4 studies overexpression of c-erbB was found in 10-17% of the tumors. Rolitsky et al. (Rolitsky et al., 1999), Reinatz et al. (Reinartz et al., 1994) and Silverman et al. (Silverman et al., 2000) found c-erbB overexpression or amplification to be strongly associated with the non-endometrioid histological subtype. Reinartz et al. (Reinartz et al., 1994) and Rolitsky et al. (Rolitsky et al., 1999) even specified these tumors to be mainly clear-cell carcinomas. However, Wang et al. (Wang et al., 1993) found the same frequen-

cies of overexpression but could not confirm the association of c-erbB expression with histological nor clinical characteristics of the tumors. Reinartz et al. (Reinartz et al., 1994) reported a much lower overexpression rate: only 2%, and found no correlation with histological features or with prognosis. Using fluorescence in situ hybridization (FISH) Riben et al. (Riben et al., 1997), and Saffari et al. (Saffari et al., 1995) found c-erbB amplification in 21%-38% of the endometrial tumors and found c-erbB amplification to be an independent prognostic factor by multivariate analysis. Patients with c-erbB amplification, as demonstrated by FISH, had lower overall survival rates, but responded better to adjuvant chemotherapy or radiation therapy than patients without c-erbB amplification. Normal and hyperplastic endometrium show a normal expression pattern of c-erbB (Rasty et al., 1998). These observations indicate that mutations in c-erbB are a late event in the carcinogenesis of endometrial cancer and may be associated with a non-endometrioid subtype and poor prognosis.

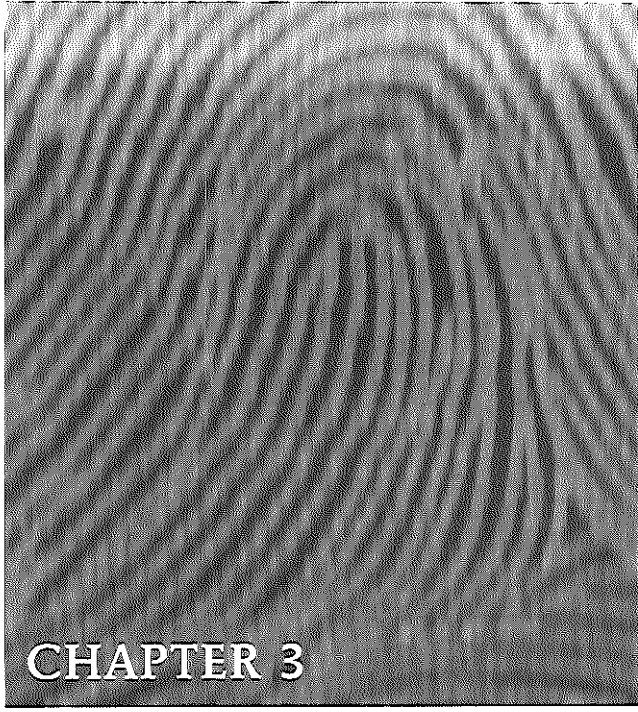
- *bcl-2* — The proto-oncogene bcl-2 is an inhibitor of programmed cell death and counteracts the action of p53, which induces apoptosis. Morsi et al. (Morsi et al., 2000) observed cyclic changes of the bcl-2 protein expression in normal endometrium, with the highest expression during the proliferative phase. Bcl-2 expression levels progressively decrease, when comparing normal endometrium with hyperplasia and carcinoma (Morsi et al., 2000). Moreover, in the endometrial carcinomas, the expression levels correlate negatively with differentiation grade (Morsi et al., 2000, Ioffe et al., 1998, Geisler et al., 1998, Zheng et al., 1996). On basis of these observations, Zheng et al. (Zheng et al., 1996) suggested that failure to inactivate bcl-2 expression is an early event in the development of endometrial carcinoma. It provides the cell with the opportunity to accumulate genetic mutations and subsequently can lead to the evolution of a precursor lesion into a carcinoma. The negative correlation of bcl-2 activity with endometrial carcinoma differentiation grade was thought to indicate that the role of bcl-2-related inhibition of apoptosis is less important in late-stage endometrial carcinoma (Zheng et al., 1996). However, Ioffe et al. (Ioffe et al., 1998) observed that not the quantity, but the subcellular localization of bcl-2 expression seems to be an important difference between normal endometrial tissues and endometrial carcinoma. In normal and hyperplastic endometrium, bcl-2 expression was mainly found in the cytoplasm, whereas in carcinomas predominantly a

nuclear localization of bcl-2 was observed. Moreover, higher proliferative rates were found in tumors with a predominantly nuclear localization as compared to tumors with a cytoplasmic bcl-2 expression pattern (Ioffe et al., 1998).

In conclusion, genes most frequently found to be altered in endometrial cancer are the tumor-suppressor genes: p53, PTEN, DCC and the oncogenes: K-ras, c-erbB2, c-fms. Two of these genes: p53 and c-erbB2, have been identified to serve as molecular prognostic markers in endometrial tumors.

2.3.4 Hormonal influences in the etiology of endometrial cancers

The endometrium is a highly hormone sensitive tissue. The cyclic changes of proliferation, differentiation and subsequently shedding of the endometrium are driven by two ovarian hormones: estradiol and progesterone. Stimulation of the endometrium by estrogens, without the differentiating effect of progestins, is the primary etiologic factor associated with the development of endometrial hyperplasia and adenocarcinoma. Endogenous (anovulation) and exogenous (hormonal therapy) sources of unopposed estrogens are generally considered as the source of increased risk of endometrioid carcinoma (Antunes et al., 1979). Also, the use of the anti-estrogen Tamoxifen (TAM) has been associated with an increased risk of endometrial cancer (Bergman et al., 2000). TAM is successfully used in the treatment of women with breast cancer. However, long-term use of TAM gives a 2-7-fold increase of the relative risk for endometrial cancer (Bergman et al., 2000, Bernstein et al., 1999). The endometrial tumor, which develops in these patients, is characterized by an association with high-stage, an aggressive histological type, overexpression of p53 and a low expression of estrogen receptors (Bergman et al., 2000, Ramondetta et al., 1998, Kuwashima et al., 1998).



CHAPTER 3

Gene expression profiles of human endometrial cancer samples using a cDNA-expression array technique: assessment of an analysis method

Gene expression profiles of human endometrial cancer samples using a cDNA-expression array technique: assessment of an analysis method.

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Summary

The recently developed cDNA expression array technique can be used to generate gene expression fingerprints of tumor specimen. To gain insight in molecular mechanisms involved in the development and progression of cancer, this cDNA expression array technique could be a useful tool. However, being a novel technique, no established methods for interpreting the results are yet available. We used the Atlas Human Cancer cDNA-expression array (Clontech, USA) for analyzing total RNA isolated from four human endometrial carcinoma samples (two cell lines and two tissue samples), one benign endometrial tissue sample and a human breast cancer cell line in order to develop a method for analyzing the array data. The obtained gene expression profiles were highly reproducible. XY-scatter plots and regression analysis of the logarithmic transformed data provided a practical method to analyze the data without the need of preceding normalization. Three genes (Decorin, TIMP3 and Cyclin D1) were identified to be differentially expressed between the benign endometrial tissue sample and the endometrial carcinoma samples (tissue and cell lines). These three genes may potentially be involved in cancer progression. A higher degree of similarity in gene expression profile was found between the endometrial samples (tissue and cell-lines) than between the endometrial samples and the breast cancer cell line, which is indicative for an endometrial tissue specific gene expression profile.

Introduction

Tumor development and progression involves a cascade of genetic alterations (Fearon & Vogelstein, 1990; Knudson, 1971). Techniques frequently used to study gene expression alterations, such as RT-PCR, differential display PCR and Northern blot analysis, have their limitations: some need large amounts of RNA, others are time consuming and can only study a small number of genes simultaneously. With the development of the cDNA expression array technique a method has become available to study the expression levels of a large range of genes in one single hybridization, requiring only a small amount of total RNA. Using this technique a gene expression fingerprint of every single tissue sample can be made. Being a novel technique, no established methods for interpreting the results are yet available. In recent publications mostly two single hybridization experiments are compared and differentially expressed genes are identified. Little attention is paid to the method that is used to analyze the data (Hoch et al., 1999; Kaiser et al., 1999; Shim et al., 1998). However, two research groups have described methods to analyze the array data: Hilsenbeck et al.(Hilsenbeck et al., 1999) introduce a statistical method to compare more than two hybridization experiments and to identify differentially expressed genes, using a principal components analysis of mean-centered log-transformed data. Rhee et al. (Rhee et al., 1999) analyses similarity in gene expression patterns between three hybridization experiments using scatter plots and rank tests.

Endometrial cancer is the most common gynecologic malignancy (Rose, 1996; Schottenfeld, 1995). In the majority of cases, approximately 75%, the tumor is confined to the uterus at time of diagnosis and has a relatively good prognosis. Patients with advanced/recurrent disease have a poor prognosis, with response rates to therapy of only 10-30% (Lentz, 1994; Rose, 1996). In general tumor development and tumor progression are thought to be driven by genetic alterations. Therefore insight into the molecular mechanisms involved in progression of endometrial carcinoma is important when searching for new tools to improve the outcome of patients with advanced/recurrent endometrial carcinomas. Using the cDNA expressing array technique, gene expression fingerprints of a variety of endometrial carcinoma tissue samples can be produced and differentially expressed genes can be identified. We used the Atlas Human Cancer Expression Array (Clontech, USA) on endometrial

carcinoma tissue and cell line samples. The analysis method and the results are evaluated in this article.

Materials and Methods

Cell lines

Ishikawa cells are derived from a human well-differentiated endometrial adenocarcinoma and were a generous gift from Dr. Masato Nishida (Tsukuba, Japan). ECC-1 cells are derived from a well-differentiated adenocarcinoma of human endometrium transplanted to nude mice and were a generous gift from Dr. P.G. Satyaswaroop (Hershey, USA). The T47D cells are derived from well-differentiated human breast cancer and were a generous gift from Dr. B. van der Burg (Utrecht, The Netherlands). All cells were maintained in DMEM/F12 (Dulbecco's modified Eagle's medium/Ham's F12 (1:1 mix) with 15mM Hepes, with L-glutamine) supplemented with penicillin/streptomycin and in the presence of 7.5% fetal calf serum and 10^{-9} M estradiol in a 37°C incubator. For the experiments, cells were cultured to a 50% confluency, medium was changed to DMEM/F12 supplemented with 7.5% dextran-coated charcoal-treated fetal calf serum and 10^{-9} M estradiol. Subsequently, cells were cultured for an other 72h before harvesting. Total RNA was isolated as described below.

Tissue samples

The endometrial carcinoma tissue samples were obtained from patients attending hospital for treatment of endometrial cancer (department of Obstetrics & Gynecology of the Erasmus M.C. Rotterdam and department of Obstetrics & Gynecology of the Saint Franciscus Hospital Rotterdam, The Netherlands). The histological type and grading were established by the department of Pathology of the Erasmus M.C. Rotterdam according to the modified FIGO staging system (Mikuta, 1993). The two human endometrial carcinoma tissue samples used were tissue sample 54: moderate well-differentiated (grade 2) endometrioid adenocarcinoma and tissue sample 55: well-differentiated (grade 1) endometrioid adenocarcinoma. The benign human endometrial epithelial tissue sample was obtained from a patient attending

the department of Obstetrics & Gynecology of the Erasmus M.C. Rotterdam for treatment of uterine myomas (tissue sample 36). The endometrial epithelial samples were excised directly after removal of the uterus from the body and frozen in liquid nitrogen. Sandwich sections were made, by the department of Pathology of the Erasmus Medical Center Rotterdam, to establish percentage of tumor in the studied samples. The carcinoma samples used in this study (sample 54 and 55) contained >90% endometrial carcinoma tissue, the benign endometrial tissue sample (sample 36) contained >90% benign endometrial epithelial tissue.

RNA isolation

Total RNA was isolated from the cell lines and the tissue specimen following the instructions of the protocol PT3231-1, advised to use in combination with the Atlas Human Cancer cDNA expression array by Clontech Laboratories, Inc. (Palo Alto, California, USA).

Atlas Human Cancer cDNA expression array

The Atlas Human Cancer cDNA expression array (7742-1) was purchased from Clontech Laboratories, Inc. (Palo Alto, California, USA). Two identical nucleic acid arrays were supplied containing 588 cancer related human cDNA's spotted as duplicates on nylon membranes. A list of the genes spotted on the array, including array coordinates, is available at Clontech's web site http://www.clontech.com.

cDNA synthesis and hybridization

Total RNA was reverse transcribed into cDNA using a mixture of array gene-specific primers and was labeled with ^{32}P-dATP. Probe purification and hybridization to the array were performed following the array's user manual. The array was exposed to a phosphor-imaging screen at room temperature for 24 hours and scanned using the Phosphor Imager (Molecular Dynamics, Sunnyvale, USA). The results were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, USA). A grid was applied to the image of the blot to quantify the intensity of the hybridization of every spot. Background signal was defined as the average of the hybridization signals pro-

duced by the nine negative controls on the array. A gene was defined to be expressed when the hybridization signal extended the value of two times the background signal. According to the Array manufacturer (Clontech Laboratories, Palo Alto, CA, USA), the radioactive cDNA signal is linear for RNAs present at levels of 0.01-3% of the total RNA population. We did not observe saturation of the hybridization signal.

Statistic analysis

- *Logarithmic transformation* — The expression data obtained from Phosphor Imager analysis were logarithmic (^{10}log) transformed.
- *XY-scatter plot* — The xy-scatter plot displays the expression levels of the genes for two compared experiments. Every dot represents one of the genes on the cDNA array. The x-value represents the ^{10}log of the expression level of the gene in one experiment, the y-value represents the ^{10}log of the expression level of the same gene in the other experiment. The xy-scatter plots were produced using Microsoft Excel 97 software.
- *Regression analysis* — The least-squares regression line $y=ax+b$ is the line with the smallest sum of squared vertical distances between the points of the xy-scatter plot and the line. The least-squared regression lines were drawn using Microsoft Excel 97 software.
- *R-squared value* — R-squared value is the Pearson correlation coefficient of the least-squares regression line. The R-squared values were calculated using Microsoft Excel 97 software.
- *Standard deviation* — The standard deviation of the distance between the points and the least-squared regression line was calculated using Microsoft Excel 97 software. An area of standard deviation multiplied with 1.96 at both sides of the least-squared regression line was drawn in the xy-scatter to establish a 95% prediction interval. Genes located outside this area were identified as differentially expressed.

Results

Using a cDNA expression array technique we established the expression profile of 588 genes selected from different areas of cancer research in two human endometrial carcinoma cell lines (Figure 3.1 A and 3.1 B), in two

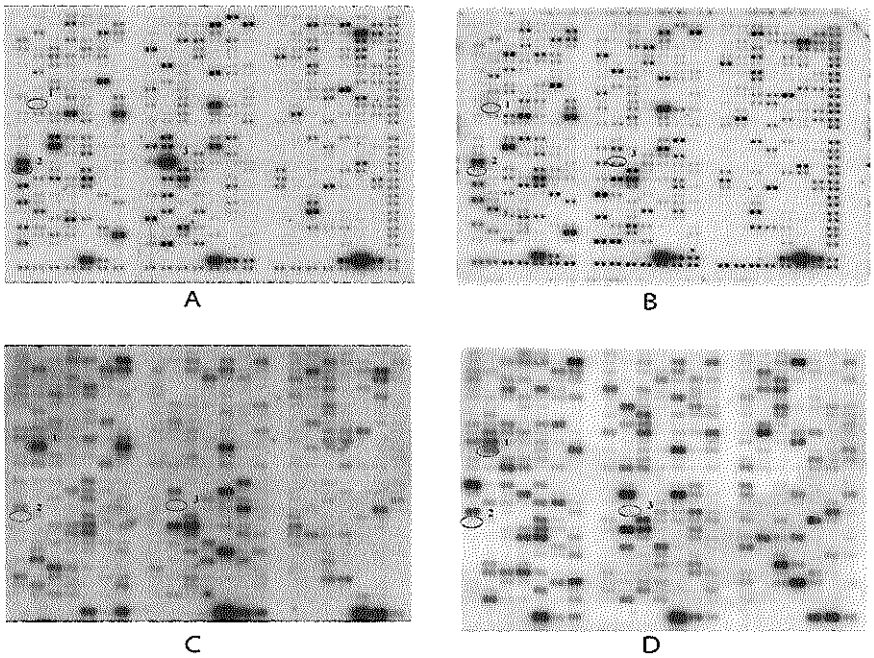

Figure 3.1: Gene expression fingerprints of endometrial cells. Atlas Human Cancer cDNA Expression Array (Clontech, USA) was hybridized with ^{32}P-labelled cDNA probes obtained from total RNA of the Ishikawa endometrial carcinoma cell-line **(Figure 3.1 A)**, the ECC-1 endometrial carcinoma cell line **(Figure 3.1 B)**, a benign human endometrial tissue sample (benign tissue sample 36) **(Figure 3.1 C)** and a well-differentiated human endometrial carcinoma tissue sample (tumor sample 54) **(Figure 3.1 D)**. The array contains 588 cancer related human cDNAs spotted as duplicates. Nine housekeeping genes are spotted at the bottom line to serve as positive controls. Dark gray spots at the outer end of the array represent genomic DNA spots, which serve as orientation marks. **1**=Cyclin D1, **2**=Decorin, **3**=TIMP3.

human endometrial carcinoma tissue samples (one shown in Figure 3.1 D), in one benign human endometrial tissue sample (Figure 3.1 C) and in one breast cancer cell line (not shown). Using logarithmic transformed raw data, obtained from phosphor imager analysis of cDNA expression array hybridization experiments, the gene expression fingerprints of the different tissue and cell line samples were compared. Using a xy-scatter plot, similarity in gene expression fingerprints between the cell lines and tissue samples was established and differentially expressed genes were identified.

Normalization of the data

It seems that comparing gene expression fingerprints between cell lines or tissue samples should be conducted only after normalization of the data for differences in background signal and for differences in intensity of hybridization. The cancer cDNA expression array contains nine genes known as housekeeping genes (liver glyceraldehyde 3-phosphate dehydrogenase, tubulin alpha, HLA class1 histocompatibility antigen C-4 α-chain, β-actin, 23-kDA highly basic protein, ribosomal protein S9, ubiquitin, phospholipase A2, HPRT). All these nine housekeeping genes, or a selection of these genes, could be used as standards in normalization. When using a xy-scatter plot and regression analysis, the raw data may be analyzed without the need of preceding normalization of the data. In the least-squares regression line $y=ax+b$ the slope value a can be considered to represent the differences in hybridization intensity and the intercept value b to represent the differences in background signal. Expression level of the highest and lowest expressed genes on the array varied extensively. Subsequently, when comparing expression data, the differences between genes with high expression levels will have much greater impact than the differences between low expressed genes. Using a logarithmic transformation of the raw data equalized these differences to some extent.

Reproducible gene expression fingerprints

Total RNA was isolated from Ishikawa cells. The RNA sample was divided in two batches, reversed transcribed and the cDNA was labeled. The two labeled cDNA batches were hybridized to two twin membranes. A very high $R^2=0.92$ was seen (Figure 2A), implying a high level of reproducibility of the cDNA array technique.

Figure 3.2: Pair-wise comparison of gene expression fingerprints using XY-scatterplot. A pair-wise comparison of the gene expression fingerprint was made between: the two Ishikawa cell hybridizations to the twin array membranes (**Figure 3.2 A**), between the benign human endometrial tissue sample 36 and the human endometrial carcinoma tissue sample 55 (**Figure 3.2 B**), between the benign human endometrial tissue sample 36 and the human endometrial carcinoma tissue sample 54 (**Figure 3.2 C**), and between the T47D breast cancer cells and the human endometrial carcinoma tissue sample 54 (**Figure 3.2 D**).

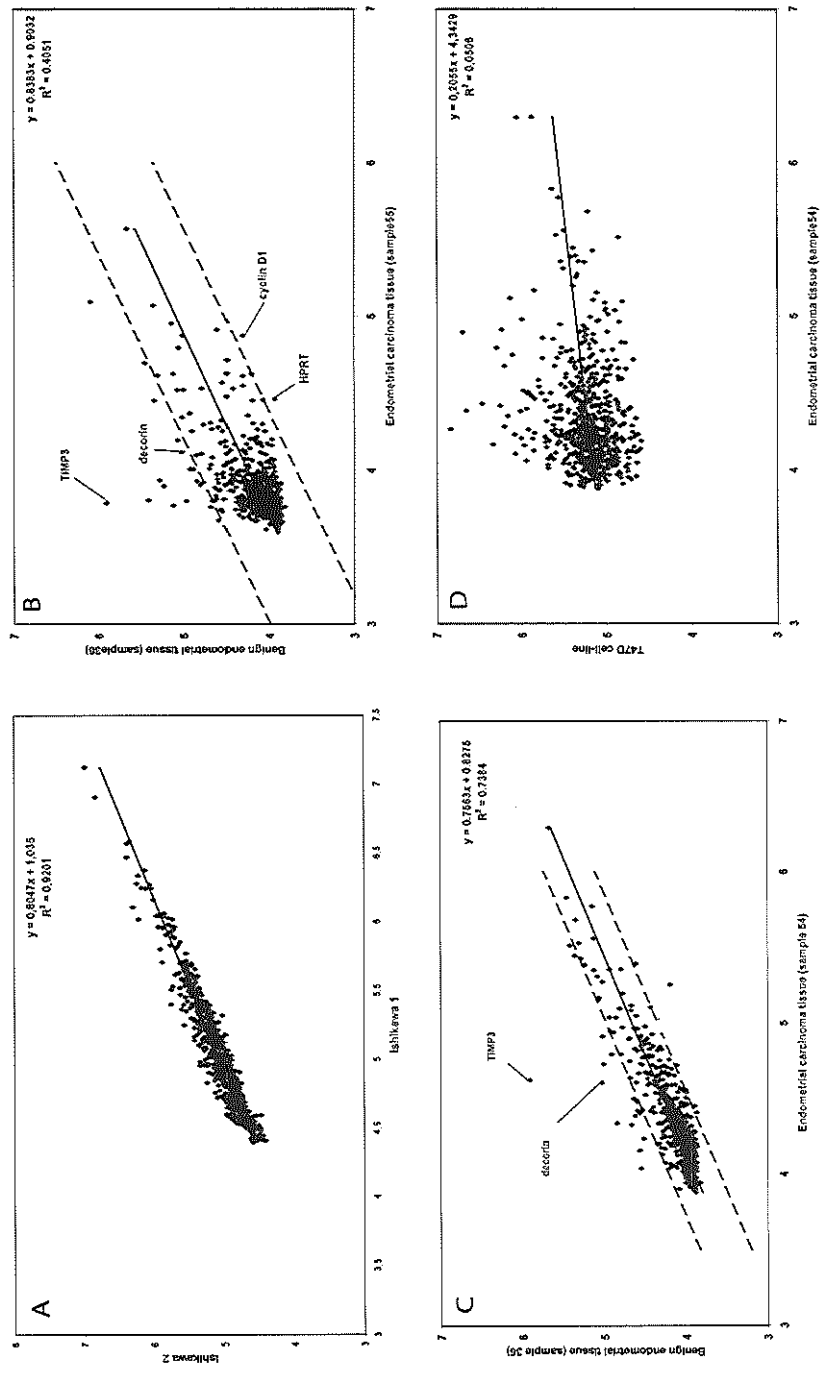
A
y = 0,8047x + 1,035
R² = 0,9201
Ishikawa 2
Ishikawa 1
B
y = 0,8383x + 0,9032
R² = 0,4051
TIMP3
decorin
cyclin D1
HPRT
Benign endometrial tissue (sample35)
Endometrial carcinoma tissue (sample55)
C
y = 0,7563x + 0,8276
R² = 0,7384
TIMP3
decorin
Benign endometrial tissue (sample 56)
Endometrial carcinoma tissue (sample 54)
D
y = 0,2055x + 4,3429
R² = 0,0508
T47D cell-line
Endometrial carcinoma tissue (sample54)

Table 3.1: Table of the R-squared values.

R-squared value	Ishikawa 1	Ishikawa 2	ECC-1	T47D	benign sample 36	tumour sample 54	tumour sample 55
Ishikawa 1		0,92	0.29	0.02	0.33	0.56	0.27
Ishikawa 2	0,92		nd	nd	nd	nd	nd
ECC-1	0.29	nd		0,33	0,22	0,26	0,42
T47D	0.02	nd	0,33		0,06	0,05	0,16
benign sample 36	0.33	nd	0,22	0,06		0,74	0,41
tumour sample 54	0.56	nd	0,26	0,05	0,74		0,47
tumour sample 55	0.27	nd	0,42	0,16	0,41	0,47	

Logarithmic transformed raw data were plotted in XY-scatter charts, regression analysis was performed and R-squared values were calculated using Microsoft Excel 97 software. nd=not determined.

Identification of similarity

All the hybridization experiments were evaluated in pairs (Figure 3.2). R-squared values (=Pearson correlation coefficient) were calculated. The R-squared value (R^2-value) represents the variation of data around the least-squares regression line. Therefore the R^2-value can be considered as a quantification of similarity between compared data. Table 3.1 gives a summary of the R^2-values. Interesting trends are seen in the R^2-values (Table 3.1). Comparing the benign endometrial tissue sample with the two endometrial carcinoma tissue samples, resulted in R^2=0,74 and R^2=0.41 respectively. A higher degree of similarity in the gene expression fingerprint was found between the endometrial samples (tissue and cell line) (average R^2=0.36 ±0.13) than between the endometrial samples (tissue and cell line) and the breast cancer cell line (average R^2=0.12 ±0.12), implicating the existence of a tissue specific gene expression fingerprint.

Specific gene expression

Using the xy-scatter plot and 95% prediction interval around the least-squares regression line, differentially expressed genes were identified. Comparing the benign endometrial tissue gene expression fingerprint with the gene expression fingerprints taken from endometrial carcinoma tissue and cell lines, three consistently differentially expressed genes were identified (Table 3.2): TIMP3 and Decorin are down-regulated in all the carcinoma samples (tissue

Table 3.2: Endometrial housekeeping genes and differentially expressed genes.

	Ishikawa	ECC-1	tumour sample 55	tumour sample 54	benign sample 36	T47D
Cyclin	x	x	x	x	x	
CD 9	x	x	x	x	x	
GRB-2 isoform	x	x	x	x	x	
c-myc binding protein	x	x	x	x	x	
c-myc transcription factor (PUF)	x	x	x	x	x	
BIGH-3	x	x	x	x	x	
Notch 2	x	x	x	x	x	
Hepatoma-derived growth factor	x	x	x	x	x	
FAU	x	x	x	x	x	
Cyclin D1; BCL-1 oncogene	x	x	x			x
Decorin					x	
TIMP3					x	

Gene expression was defined as a hybridisation signal extending the value of two times the background signal and are represented by x.

samples and cell lines), Cyclin D1 is up-regulated in the carcinoma samples, except for the endometrial carcinoma tissue sample 54 (Figures 3.2 B and 3.2 C). These three genes may represent important genes involved in the progression of endometrial carcinomas. Another group of genes, which showed expression at similar levels in all endometrial samples, was identified (Table 3.2). None of these genes was expressed in the human breast cancer cell line T47D. These genes seem to represent endometrial housekeeping genes. Comparing the breast cancer cell line T47D with the endometrial carcinoma samples (tissue and cell line) (Figure 3.2 D) revealed too many differences (R^2=0.12 ±0.12; Table 3.1) in gene expression pattern; therefore identification of differential expressed genes was not possible (see discussion).

Discussion

The expression level of all the 588 genes on the cDNA expression array plus the 21 positive and negative control dots were used in the analysis. The majority of the gene expression level signals did not exceed two times the background hybridization signal level (Figure 3.1). In the xy-scatter plot the

least-squares regression line will be highly determined by these signal levels. As long as a high degree of similarity exists between the expression pattern of the array experiments, the slope value a (in the least-squares regression line $y=ax+b$) can be considered to represent the differences in hybridization intensity and the intercept value b to represent the differences in background signal. Therefore, raw data, obtained from cDNA expression array hybridization experiments, can be analyzed without the need of preceding normalization of the data (Figure 3.2 A, 3.2 B, 3.2 C). In the same instance differentially expressed genes can be identified using a 95% prediction interval around the least-squares regression line. The R^2-value of the least-squares regression line quantifies the degree of similarity. However, when little similarity exists between the compared expression patterns, resulting in a low R^2-value, the least-squares regression line will be almost randomly chosen. Example of this situation is seen in the comparison between the gene expression fingerprint of the T47D cell line and the endometrial carcinoma tissue sample (Figure 3.2 D). Differentially expressed genes cannot be identified in this case. The lowest R^2-value at which the least-squares regression line and 95% prediction interval around the regression line can be used to identify differentially expressed genes has yet to be established.

A higher degree of resemblance exits between the endometrial samples (tissue and cell line samples), than between the endometrial samples and the breast cancer cell line, implicating a basic endometrial gene expression fingerprint. As tumor behavior is thought to be driven by genetic alterations, it is plausible that clinical behavior of carcinomas can be reflected in their specific gene expression fingerprints (Blok et al., 1999; Blok et al., 1995; Chang et al., 1997). Taking this one step further, a database could be constructed containing gene expression fingerprints of various endometrial carcinoma tissue samples of different clinical stage, different histological grade and different progesterone and estrogen receptor status linked to information on clinical follow-up. Comparing the gene expression fingerprint of an uncharacterized tumor sample of interest with the database could reveal the potential clinical behavior of the uncharacterized tumor. A table of R^2-values, as shown in Figure 3.2, could be used to establish the degrees of similarity between the tumor samples. Using the cDNA expression array to reveal degrees of similarity between uncharacterized tumor samples of interest and tumor samples of known clinical behavior could have great potential value in the diagnosis and prognosis of endometrial cancer. However, to confirm

the potential value of the cDNA arrays as a diagnostic and prognostic tool, a gene expression fingerprint database of a large group of fully characterized endometrial carcinoma tissue samples should be constructed and analyzed. The current investigation may provide a practical and useful method to analyze these data.

Comparing gene expression fingerprints of different tumor samples will also identify differentially expressed genes. Using the xy-scatter plot analysis and the 95% prediction interval around the least-squares regression line, three genes (Decorin, TIMP3 and Cyclin D1) were identified to be differentially expressed between benign endometrial cells and endometrial carcinoma cells. Decorin (small leucin rich protoglycan) inhibits TGFβ and induces p21 resulting in inhibition of proliferation (Iozzo et al., 1999; Stander et al., 1999). TIMP3 (tissue inhibitor of metaloproteinase number 3) inhibits the matrix metaloproteinases MMPs. Excess of MMPs stimulates tumor invasion and metastasis (Kugler, 1999; Sato et al., 1992). Cyclin D1 initiates the G1-S phase progression (Chen et al., 1998). Estrogens are found to induce nuclear localization of Cyclin D1 in endometrial cells resulting in increased cell proliferation (Tong & Pollard, 1999). In breast cancers Cyclin D1 can act as an oncogene (Russell et al., 1999) and is found to act as a CDK-independent activator of the estrogen receptor in breast epithelial cells (Zwijsen et al., 1997). The loss of Decorin and TIMP3 expression and the gain of Cyclin D1 expression in the endometrial carcinoma cell lines and tissue samples could indicate an important role of Decorin, TIMP3 and Cyclin D1 in the development and progression of endometrial cancer. However, the number of samples analyzed is too small to draw conclusions.

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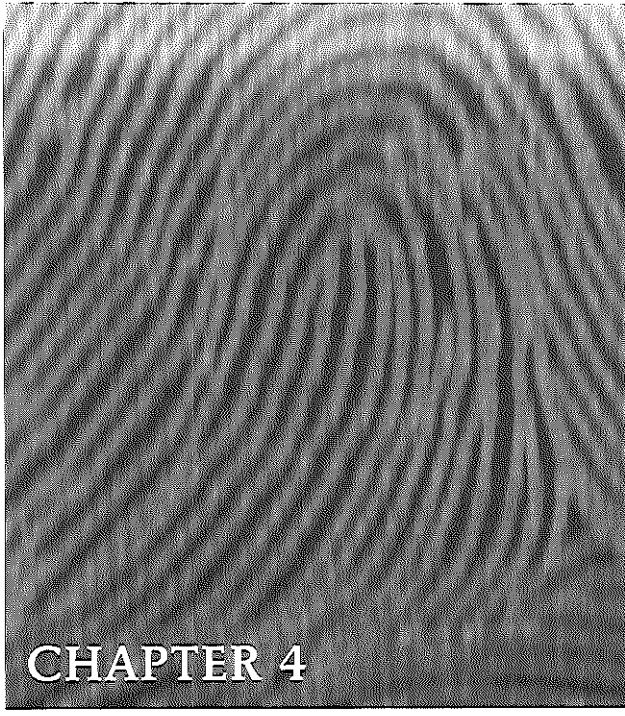

CHAPTER 4

Gene expression profiling of human endometrial cancer tissue samples: utility and diagnostic value

Gene expression profiling in human endometrial cancer tissue samples: utility and diagnostic value.

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Submitted

Summary

Recently, gene expression profiling techniques have been used on several human cancers to classify tumor-subgroups with specific biological behavior, which were previously undetected by the conventional histopathologic staging systems. In the current study the clinical usefulness and diagnostic value of gene expression profiling in human endometrial carcinomas was studied. The Atlas Human Cancer cDNA array, containing cDNAs of 588 genes selected from different areas of cancer research, was used to generate gene expression profiles of tumor tissue samples. The gene expression profiles of twelve endometrial cancers, three benign (e.g. non-cancer) endometrial tissue samples and three myometrial tissue samples, taken from human surgical specimen, were compared. The efficacy to generate a gene expression profile of these tissue samples was 77%. The RNA samples could be randomly taken from the tissue samples and were highly reproducible. Cluster analysis of the gene expression profiles of the different samples showed that the benign endometrial and the myometrial samples clustered separately from the tumor samples, indicating that the gene expression profiles were tissue specific and not patient specific. Cluster analysis of the tumor samples revealed two distinct tumor clusters. The ranking of the tumors in the two clusters showed high similarity with the histopathologic classification (FIGO grading), indicating that the diagnostic value of classification of endometrial tumors on basis of their gene expression profiles can be at least as accurate as the FIGO grading system.

INTRODUCTION

Endometrial cancer ranks among the most common gynecologic malignancies in the Netherlands and North America, alongside ovarian cancer (Bristow, 1999) (Central Office of Statistics of the Ministry of Public Health of The Netherlands 1999). In order to predict prognosis and to select optimal therapeutic regimes, endometrial carcinomas are divided into subgroups. A surgical and histological staging system of the International Federation of Gynecology and Obstetrics (FIGO) (Pecorelli et al., 1999)(http://www.figo.org) is at present the golden standard. However, patients with the lowest surgical-pathological stage, stage 1, have a 5-years survival rate of 83%, indicating that 17% of stage 1 patients are misguidedly taken to have a good prognosis (Abeler & Kjorstad, 1991). Moreover, several studies have shown that the prognosis of patients with endometrial cancer is correlated with various factors other than FIGO stage. Morphometric nuclear grade (Salvesen et al., 1998), lymph-vascular space invasion (Feltmate et al., 1999), DNA ploidy (Evans & Podratz, 1996; Lim et al., 1999), estrogen/progesterone receptor status (Creasman, 1993) and p53 overexpression (Soong et al., 1996) have been shown to be independent prognostic factors.

With the development of the cDNA array technology, a method has become available to study the expression levels of a large range of genes in one hybridization, requiring only a small amount of RNA. Using this technique, a gene expression profile of a single tissue sample can be made. Comparisons of gene expression profiles have been performed in several different human cancers. These studies show that gene expression profiles can identify tumor-subgroups with specific biological behavior, which were previously undetected by the histopathologic staging systems (Alizadeh et al., 2000; Alon et al., 1999; Perou et al., 2000; Shim et al., 1998; Sorlie et al., 2001).

Integration of factors related to the biological behavior of the tumor, such as gene expression profiles, in order to facilitate an estimation of individual prognosis seems promising and may lead to a more accurate classification system and better therapeutic regime of cancers. So far, the recent study of Mutter et al., (Mutter et al., 2001) is the only report on gene expression profiling in endometrial cancer . Using an Affymetrix GeneChip array, they have found that gene expression patterns of endometrial carcinomas resemble proliferative rather than secretory normal endometrium. However, neither the issue of the diagnostic value of gene expression profiling in endometrial

carcinomas nor the feasibility of gene expression profiling techniques in a clinical setting was addressed by Mutter et al., (Mutter et al., 2001). In the current study we investigated the utility and diagnostic value of gene expression profiling in human endometrial cancers.

Materials and Methods

Tissue samples

Between 1997 and 2001 endometrial carcinoma tissue samples were obtained from patients attending hospital for treatment of endometrial cancer (department of Obstetrics & Gynecology of the Erasmus Medical Center Rotterdam and department of Obstetrics & Gynecology of five affiliated hospitals (Saint Franciscus Hospital Rotterdam, Medical Center Rijnmond Zuid (location Clara) Rotterdam, Ikazia Hospital Rotterdam, Reinier de Graaf Group Delft and Albert Schweitzer Hospital Dordrecht), following approval by the human subjects review boards of the participating hospitals. When possible, benign endometrial and myometrial samples were taken from the same patients. Also benign endometrial samples from hysterectomy material of patients with uterine myomas or menstrual bleeding disorders without a uterine carcinoma were collected. The histological type and grading were established on the hysterectomy specimen, by the department of Pathology of the participating hospitals, according to the modified FIGO staging system (Pecorelli et al., 1999)(http://www.figo.org). Directly after removal of the uterus from the body, tissue samples with macroscopically high percentage of the tissue of interest (tumor, benign endometrium or myometrium) were located, excised and flash-frozen in liquid nitrogen. Sandwich sections were made of these flash-frozen samples. The parts of the flash-frozen samples in between the sandwich sections were used for total RNA isolation. The sandwich sections were hematoxylin and eosin stained to analyze microscopically the percentage of tumor, benign endometrial or myometrial cells in the studied samples. The percentage was established by placing a 100-point grid on representative areas of the sandwich sections, and identifying the cell type of the tissue fragment underlying those points. Only tissue samples containing more than 80% of the indicated tissue were used in this study.

RNA isolation

Total RNA was isolated from the tissue specimen following the instructions of the protocol PT3231-1, advised to use in combination with the Atlas Human Cancer cDNA expression array by Clontech Laboratories, Inc. (Palo Alto, California, USA). The quality of the total RNA (intact RNA) was judged on northern blot by ethidium bromide staining.

Atlas Human Cancer cDNA expression array

The Atlas Human Cancer cDNA expression array (7742-1) was purchased from Clontech Laboratories, Inc. (Palo Alto, California, USA). cDNA arrays were supplied containing 588 cancer related human cDNA's spotted as duplicates on nylon membranes. A list of the genes spotted on the array, including array coordinates, is available at Clontech's web site http://www.clontech.com. Ten arrays were used, derived from two different lots. Each array was stripped and re-used at a maximum of three times.

cDNA synthesis and hybridization

Total RNA was reverse transcribed into cDNA using a mixture of array gene-specific primers and was labeled with ^{32}P-dATP. Probe purification and hybridization to the array was performed following the array's user manual. The array was exposed to a phosphor-imaging screen at room temperature for 24 hours and scanned using the Phosphor Imager (Molecular Dynamics, Sunnyvale). The results were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale). A grid was applied to the image of the blot to quantify the intensity of hybridization of every spot. According to the Array manufacturer (Clontech Laboratories, Palo Alto, CA), the radioactive cDNA signal is linear for RNAs present at levels of 0.01-3% of the total RNA population. Saturation of the hybridization signal was not observed.

Analysis of the cDNA expression array data

Reproducibility of hybridization
Hybridization of one cDNA pool to two different array membranes showed that gene expression profiles, obtained using the cDNA array technique, are

highly reproducible (Smid-Koopman et al., 2000).

Cluster analysis

The Cluster® software, a hierarchical cluster algorithm, was used to compare the gene expression patterns of the different tissue samples. The Cluster and Tree View® software is created by Michael Eisen et al (Eisen et al., 1998) and is available at http://rana.stanford.edu/software. This cluster algorithm is an unsupervised pair-wise average-linkage cluster method and clusters both genes and arrays (the algorithm is described in a paper by Michael Eisen et al (Eisen et al., 1998) and in the manual provided with the software at http://rana.stanford.edu/software). The complete linkage clustering for both genes and patients was used. No filter was applied. To compensate for possible variations in overall hybridization intensity between the different arrays, the intensity of expression level of each cDNA on each array was multiplied by the quotient of the sum of expression levels of all genes on that array with the average sum of expression levels of all genes for all the arrays.

Results

Utility of the Atlas Human Cancer cDNA expression array on human endometrial cancer tissue samples

To examine the feasibility of gene expression profiling as a clinical tool, we studied the efficacy, reproducibility and specificity of gene expression profiles of human tissues obtained from surgical specimen. We used a commercial available macro cDNA array, as the costs are relative low and the technique relatively easy to apply.

Efficacy

Materials from a human tissue bank, containing endometrial carcinoma, myometrial and benign endometrial tissue samples (see Materials and Methods), were used to generate gene expression profiles of the indicated human tissues. Sandwich sections were made of these samples to establish the percentages of tumor tissue, benign endometrial epithelium and myometrium tissue in the samples. 88% of the tested samples appeared to contain >80% of the indicated tissue, and were subsequently used to isolate total RNA. Of

these samples 94% generated quantitative and qualitative good (intact) total RNA to be used on the Atlas Human Cancer cDNA Array. Generally, 50 mg tissue (approximately 15 sections of 30 μm) generated 30 μg total RNA. One hybridization to an Atlas Human Cancer cDNA array membrane required approximately 10 μg total RNA. The efficacy to perform successful hybridization to the array membrane could, in our hands, be optimized to 93%. All together, the efficacy to generate a gene expression profile from a sample taken from a surgical specimen was found to be 77% and appeared to be mainly determined by the efficacy of tissue sampling from the surgical specimen.

Reproducibility

The tissue bank contained tissue samples from surgical specimen. Macroscopically judged, samples containing high percentage of the indicated tissue were excised from the surgical specimen and flash-frozen in liquid nitrogen. Sections were randomly taken from these tissue samples to isolate RNA. To determine whether a gene expression profile, characteristic for an individual tumor (or tissue), can be generated from randomly chosen sections taken from a tumor (or tissue) sample, two different sections were taken from the same tumor sample (patient 42). Total RNA was isolated from these two sections and hybridized to two different array membranes. The gene expression pattern of the nineteen different tissue samples were compared and subsequently clustered using a hierarchical cluster algorithm (Cluster® software) (Figure 4.1). The two sections from the tumor of patient 42 clustered together and showed the highest correlation coefficient. This indicates that a gene expression profile, specific for an individual tumor, can be generated from different, randomly chosen, parts of the tumor.

Specificity

Gene expression profiles generated from the same patient but from different tissues, e.g. endometrial carcinoma vs. myometrium (Figure 4.1: patients 48, 55, and 59) did not cluster together. This indicates that the gene expression profiles generated were tissue specific and not patient specific.

Classification of human endometrial tumor samples using cluster analysis on their gene expression profile

To study the possible prognostic value of gene expression profiling in hu-

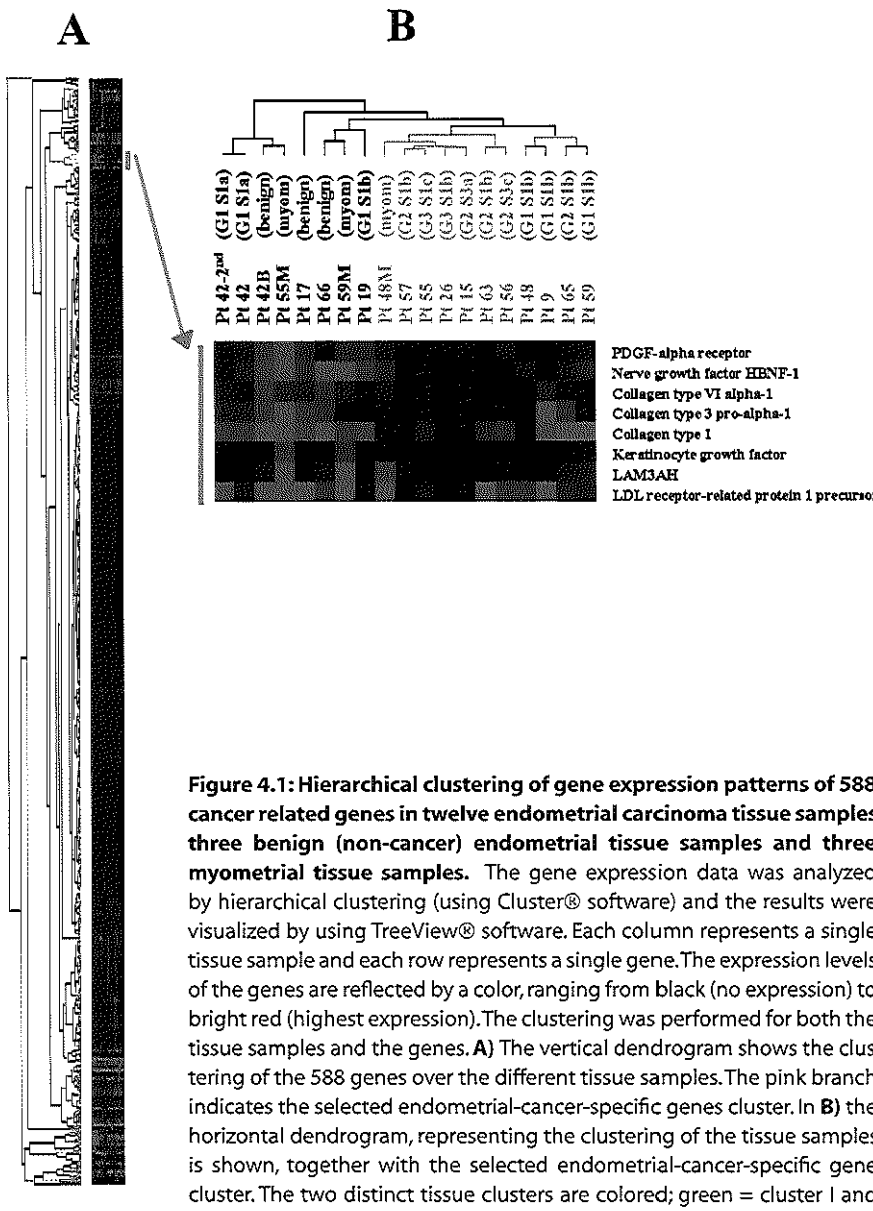


Figure 4.1: Hierarchical clustering of gene expression patterns of 588 cancer related genes in twelve endometrial carcinoma tissue samples, three benign (non-cancer) endometrial tissue samples and three myometrial tissue samples. The gene expression data was analyzed by hierarchical clustering (using Cluster® software) and the results were visualized by using TreeView® software. Each column represents a single tissue sample and each row represents a single gene. The expression levels of the genes are reflected by a color, ranging from black (no expression) to bright red (highest expression). The clustering was performed for both the tissue samples and the genes. **A)** The vertical dendrogram shows the clustering of the 588 genes over the different tissue samples. The pink branch indicates the selected endometrial-cancer-specific genes cluster. In **B)** the horizontal dendrogram, representing the clustering of the tissue samples, is shown, together with the selected endometrial-cancer-specific gene cluster. The two distinct tissue clusters are colored; green = cluster I and red = cluster II. Each tissue sample has been numbered (patient number), and the nature of the tissue sample and of the tumor samples are mentioned (benign = non-cancer endometrial tissue, myom = myometrial tissue, G = Histological Grade of the endometrial carcinoma tissue (FIGO Staging system), S = Surgical Stage of the endometrial carcinoma tissue (FIGO Staging system)).

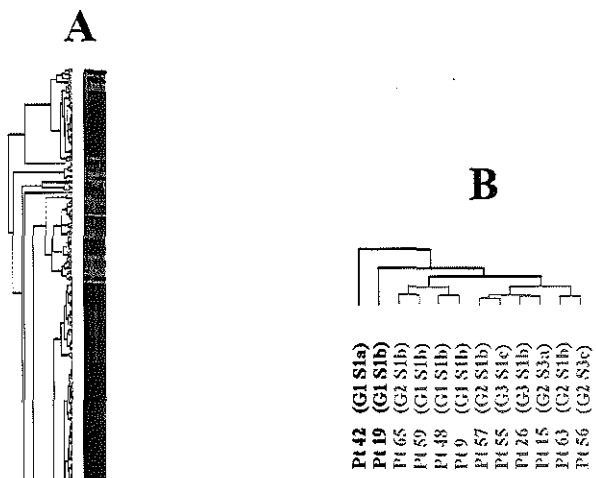


Figure 4.2: Hierarchical clustering of gene expression patterns of 588 cancer related genes in twelve endometrial carcinoma tissue samples and three benign (non-cancer) endometrial tissue samples. A) The vertical dendrogram shows the clustering of the 588 genes over the different tumor samples. The pink branch indicates the selected endometrial-cancer-specific genes cluster. In **B)** the horizontal dendrogram, representing the clustering of the tissue samples, is shown, together with the selected endometrial-cancer-specific gene cluster. The two distinct tissue clusters are colored; green = cluster I and red = cluster II. Each tissue sample has been numbered (patient number), and the nature of the tissue sample and of the tumor samples are mentioned (benign = non-cancer endometrial tissue, G = Histological Grade of the endometrial carcinoma tissue (FIGO Staging system), S = Surgical Stage of the endometrial carcinoma tissue (FIGO Staging system)).

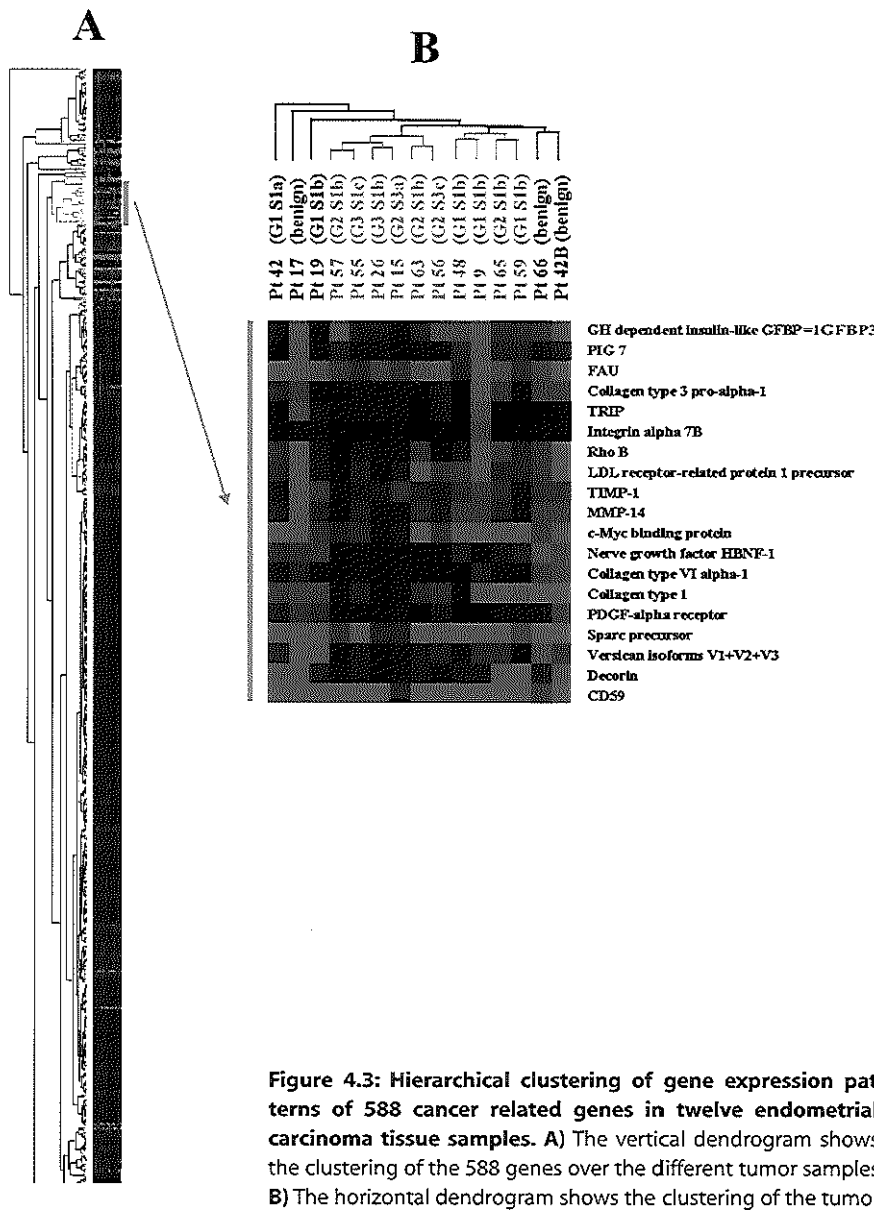


Figure 4.3: Hierarchical clustering of gene expression patterns of 588 cancer related genes in twelve endometrial carcinoma tissue samples. A) The vertical dendrogram shows the clustering of the 588 genes over the different tumor samples. **B)** The horizontal dendrogram shows the clustering of the tumor samples. The two distinct tumor clusters are colored; green = cluster I and red = cluster II. The tumor samples: patient 42 and patient 19 are not colored (black) as they did not belong to any cluster. Each tumor sample has been numbered (patient number), and the FIGO Grade and Stage is mentioned (G = Histological Grade, S = Surgical Stage).

Table 4.1. Clinicopathologic features of the tissue samples.

	Age (years)	Tissue type	Grade	Stage	Follow-up (years)
Patient 9	75	Endometrioid adenocarcinoma	1	1B	3½
Patient 15	73	Endometrioid adenocarcinoma	2	3A	3
Patient 17	41	Benign / uterine myomas	-	-	-
Patient 19	97	Endometrioid adenocarcinoma	1	1B	3
Patient 26	77	Endometrioid adenocarcinoma	3	1B	2½
Patient 42	53	Endometrioid adenocarcinoma	1	1A	2
Patient 42B	53	Benign endometrium epithelium	-	-	-
Patient 48	71	Endometrioid adenocarcinoma	1	1B	2
Patient 48M	71	Myometrium	-	-	-
Patient 55	63	Endometrioid adenocarcinoma	3	1C	1½
Patient 55M	63	Myometrium	-	-	-
Patient 56	71	Papillary serous adenocarcinoma	2	3C	1½
Patient 57	72	Papillary serous adenocarcinoma	2	1B	1½
Patient 59	67	Endometrioid adenocarcinoma	1	1B	1½
Patient 59M	67	Myometrium	-	-	-
Patient 63	71	Endometrioid adenocarcinoma	2	1B	1
Patient 65	69	Endometrioid adenocarcinoma	2	1B	1
Patient 66	43	Benign endometrium epithelium	-	-	-

Age is the age of the patient at time of surgery. *Tissue type* indicates the histopathologic evaluation of the tissue samples. *Grade* indicates the histological grade of the tumors according to the modified FIGO staging system (Pecorelli et al., 1999) (http://www.figo.org). *Stage* indicates the surgical stage of the tumors according to the modified FIGO staging system. *Follow-up* indicates the years of clinical follow up. – indicates not applicable.

man endometrial cancers, we used a hierarchical cluster algorithm; Cluster® software (available at http://rana.stanford.edu/software) to sort the tumor samples based their gene expression pattern.

Classification of the tumor samples

The Cluster® analysis revealed two distinct tumor clusters (I = green and II = red) (Figure 4.2). Two tumor samples did not belong to any cluster (patient 42 and patient 19 = black). The division of the tumors between the two clusters showed high similarity with the histopathologic classification (FIGO grading) of the tumors (Table 4.1, Figures 4.1-4.3). Cluster II (red) includes Grade 2 and Grade 3 tumors, whereas cluster I (green) contains almost exclusively Grade 1 tumors (except for patient 65 which has been classified as a Grade 2 tumor) (Figure 4.2). The two separate placed tumors (patient 42 and patient 19; black) are both Grade 1 tumors. Remarkable, the Grade 2 tumors in cluster B (red) appear to be either of papillary serous histologi-

cal subtype (patient 56 and 57) and/or of a high surgical stage (patient 15) (with exception of patient 63; being a Grade 2 Stage 1b tumor). Moreover, the Grade 2 tumor (patient 65) in cluster I (green) is of a low surgical stage. These results seem to indicate that the two cluster groups represent tumors with specific clinical behavior: cluster II (red) containing tumors with a less favorable prognosis compared to the cluster I (green).

The tumor samples of patient 19 and patient 42 (black) had the lowest correlation coefficient with the other tumors in the "only tumors" clustering (Figure 4.2). In the "all samples" clustering (Figure 4.1) these two tumor samples (patient 19 and 42, black) did not belong to any cluster, neither did the benign endometrium or the myometrium samples.

So far, the follow-up of the patients (Table 4.1) has been to short (0-3½ years) to draw conclusions about the prognostic value of gene expression profiling in endometrial cancers. Nevertheless, the present results indicate that classification of endometrial tumors using gene expression profiles highly resemble classification on basis of the FIGO grading system.

Stability of the classification

When entering the benign samples in the clustering, all these benign samples clustered outside the tumor cluster and moreover, the tumor clustering was not altered (Figure 4.3). Even more, when entering also the myometrium samples in the clustering, the tumor clustering was not altered either (Figure 4.1). This observation supports the stability of the classification.

The fact that the benign and myometrial samples cluster apart from the tumor samples is a further support for the tissue specificity of the gene expression profiles.

Identifying endometrial carcinoma specific gene clusters

To identify genes possibly involved in the development and/or progression of endometrial carcinoma, Cluster® analysis was performed on the genes; sorting the genes on basis of their expression pattern in the different tumor samples and in the benign and myometrial samples. One endometrial-cancer-specific gene cluster was identified, containing eight genes down-regulated in most tumor samples compared to the benign and myometrial samples (Figure 4.1). This cluster consists of genes involved in cellular growth regulation or in controlling cell motility and cell invasion. One of the genes in this

cluster is the PDGF-alpha receptor. Down-regulation of this PDGF-alpha receptor is known to be important for creating the immortalized phenotype of human cancer cells.

Sorting the genes on basis of their expression pattern in the different tumor and benign samples but excluding the myometrium tissue from the cluster analysis revealed that the PDGF-alpha receptor is part of an even bigger gene cluster (Figure 4.3). This gene cluster consists of nineteen genes, all involved in cellular growth regulation or in controlling cell motility and cell invasion. Even more, this cluster contained 75% of the genes of the earlier mentioned gene cluster (Figure 4.1), supporting the suggested specificity of these genes for endometrial cancer tissue.

It was not possible to identify one or more genes specific for one of the tumor subgroups (Figure 4.2).

Discussion

In the current study it is shown that, using a commercially available cDNA array technique, which is relatively low in cost and easy to use, it is possible to generate a tissue specific gene expression profile from surgical specimen in 77% of the cases. This indicates the possible utility of gene expression profiling in a clinical setting. The efficacy to generate a gene expression profile of a tumor appeared to be mainly determined by the efficacy of the tissue sampling from the surgical specimen.

To interpret the possible biological information present in the gene expression patterns, we analyzed the gene expression data using a hierarchical cluster algorithm. Cluster analysis is an exploratory data analysis tool to sort cases (people, things, events, etc) into groups, or clusters, in which the degree of association is strong between members of the same cluster and weak between members of different clusters. Cluster analysis is used in many different fields of research (e.g. economics, social science and biology). Several molecular biology research groups have used the cluster analysis in combination with gene expression profiling to classify tumors (Alizadeh et al., 2000; Alon et al., 1999; Perou et al., 1999; Perou et al., 2000; Sorlie et al., 2001; van 't Veer et al., 2002). Moreover, it was found that classification based on cluster analysis of gene expression patterns can be used as a prognostic marker with respect to overall and relapse-free survival in patients, even in

a subset of patients that was classified in the same group using traditional histological classification systems (Alizadeh et al., 2000; Sorlie et al., 2001; van 't Veer et al., 2002). Molecular classification of tumors on the basis of gene expression may thus identify previously undetected but clinically significant subtypes of cancer. The current study is the first report addressing the diagnostic and prognostic value of gene expression profiling in endometrial carcinomas and shows that endometrial tumor clusters, identified by gene expression profiling, highly resembles the FIGO grading system. However, whether gene expressing profiling is more accurate in predicting prognosis in endometrial carcinomas compared to the FIGO classification system is not yet possible to determine. So far, the follow-up of the patients studied is too short and the group to small to draw conclusions.

Using gene expression profiling, some researchers have been able to identify tissue specific gene clusters (Alizadeh et al., 2000; Golub et al., 1999; Mutter et al., 2001; Perou et al., 1999; Perou et al., 2000). In endometrial carcinomas Mutter et al., (Mutter et al., 2001), using an Affymetrix GeneChip array, have found gene expression patterns of endometrial tumors to resemble proliferative rather than secretory normal endometrium. In the current study an endometrial-cancer-specific gene cluster was identified. The genes in this cluster were down-regulated in the endometrial cancer tissues compared to the normal endometrial tissues.

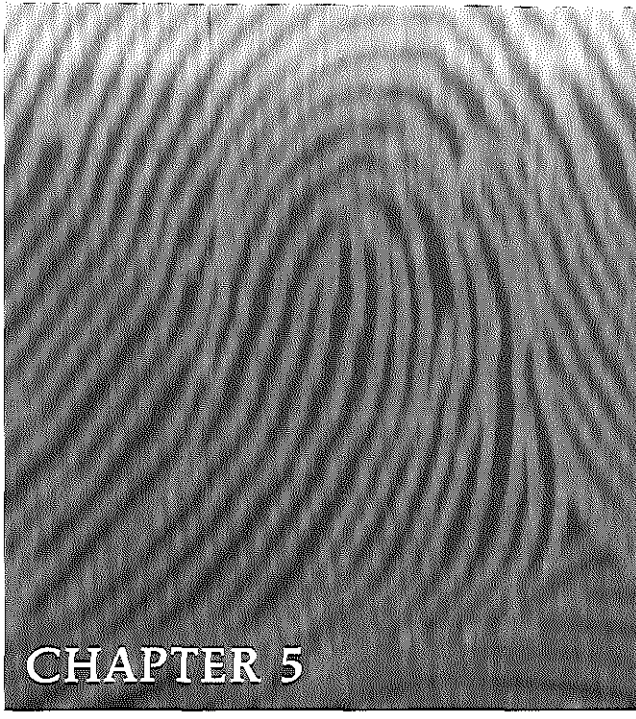
Two genes in the endometrial-cancer-specific gene cluster are of special interest, as they are known to be progesterone regulated (insulin-like growth factor binding protein 3 (IGFBP3) and integrin alpha 7B). Endometrium is a highly hormonal sensitive tissue; proliferation is stimulated by estrogens, while progesterone inhibits proliferation and stimulates differentiation (Clemens et al., 1979; Siiteri et al., 1977; Song & Fraser, 1995). Because of its ability to induce differentiation, progesterone can be used to suppress growth of endometrial cancer cells (Ehrlich et al., 1981). We have found IGFBP3 and integrin alpha 7B to be progesterone regulated in specific endometrial cancer sub-cell lines (IGFBP3 was down-regulated by progesterone in an Ishikawa sub-cell line expressing only progesterone receptor B and integrin alpha 7B was up-regulated by progesterone in an Ishikawa sub-cell line expressing only progesterone receptor A) (Smid-Koopman et al., in preparation). Bicsak et al. (Bicsak et al., 1990) found IGFBP3 to inhibit granulosa cell proliferation in rat ovaries and subsequently to inhibit estradiol and progesterone production by the granulosa cells . Down-regulation of IGFBP3 has been found to inhibit

apoptosis (Baxter, 2001; Butt et al., 1999; Grimberg & Cohen, 1999; Shen et al., 1999). Furthermore, IGFBP3 down-regulation has also been correlated with worst prognosis in ovarian cancer (Katsaros et al., 2002). Decline of integrin expression has more frequently been found in poorly differentiated endometrial cancers and is associated with metastatic nodal spread (Lessey et al., 1995).

In the current study, it was not possible to find gene clusters associated with histological grade or clinical stage of the tumors. This may be due to the fact that, in contrast to the earlier mentioned studies (Alizadeh et al., 2000; Golub et al., 1999; Perou et al., 1999; Perou et al., 2000) which used micro-arrays containing thousands of genes, the current study used a selected group of only 588 genes. Furthermore, only a small number of tumor samples were used in the current study. Increasing the number of endometrial tumor samples to generate expression profiles will probably increase the chance of finding specific clusters associated with a certain histological grade.

In conclusion, the current study has established the clinical feasibility of the cDNA array technique to generate gene expression profiles of endometrial carcinoma tissue samples. The prognostic value of classification of endometrial tumors on basis of their gene expression pattern is at least as powerful as the FIGO grading system. Classification of endometrial tumors on basis of their gene expression pattern seem to have the potential to serve as a classification system more accurate in predicting the biological behavior of the tumors than the histopathologic FIGO classification. However, characterization of the gene expression profile of a large number of endometrial tumors, combined with a long-term clinical follow-up, is needed to confirm this hypothesis.

Acknowledgements
We are grateful to medical staff of the department of Gynecology and of the department of Pathology of the participating clinics (Dijkzigt Hospital, Erasmus M.C. Rotterdam, Saint Franciscus Hospital Rotterdam, Medical Center Rijnmond Zuid (location Clara) Rotterdam, Ikazia Hospital Rotterdam, Reinier de Graaf Group Delft and Albert Schweitzer Hospital Dordrecht) and to the participating women for the gift of human endometrial carcinoma tissue samples. This work was supported by a grant from The Netherlands Organization for Scientific Research (NWO 903-46-169) and by the Erasmus Trust Funds.



CHAPTER 5

Differential gene expression in progesterone sensitive and progesterone insensitive endometrial carcinoma cells

Differential gene expression in progesterone sensitive and progesterone insensitive endometrial carcinoma cells.

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Summary

High doses of progesterone are used in the treatment of advanced and recurrent endometrial cancer. Unfortunately the response rate is relatively low: 10-30%. The mechanisms involved in the development of insensitivity to progesterone treatment of endometrial cancer tissue are largely unknown. As tumor development is thought to be associated with a cascade of genetic alterations, it can be expected that genetic changes are involved in the development of progesterone insensitivity in endometrial carcinomas. We therefore started an investigation to identify, isolate and characterize progesterone-regulated genes involved in progesterone induced growth inhibition in endometrial carcinoma cells. Using differential display PCR eight progesterone regulated cDNA clones were identified in endometrial carcinoma cell lines. Four of these progesterone regulated cDNA clones were regulated in the for growth progesterone sensitive cell line IK-3H12 and not regulated in the for growth insensitive cell line ECC-1. This indicates that these four cDNA clones represent potentially important genes, which could be involved in inhibition of growth of endometrial carcinoma tissue by progesterone.

Introduction

Endometrial cancer is the most common gynecologic malignancy, and is usually diagnosed at an early stage (Schottenfeld, 1995). Standard therapy in these cases includes abdominal hysterectomy with bilateral salpingo-oophorectomy, sometimes followed by radiotherapy. In most patients this therapy is curative (Maneschi et al., 1992). However patients with recurrent disease after treatment or patients who have an advanced disease at the beginning of treatment (FIGO stage III/IV), have a poor prognosis. Hormonal treatment is optionally given to these patients, with response rates of only 10-30% (Lentz, 1994). In contrast to hormonal treatment of advanced/recurrent endometrial carcinomas, the treatment of benign functional endometrial disorders and endometrial hyperplasia with progestins is very successful (Lindahl & Willen, 1991). It seems that at some stage in the progression of endometrial carcinomas, sensitivity to progesterone treatment is lost. The transition from hormone sensitive to hormone insensitive tumor growth may be associated with genetic alterations (e.g. activation of oncogenes and/or inactivation of tumor suppressor genes) (Fearon & Vogelstein, 1990; Knudson, 1993). In order to obtain more insight into the molecular mechanisms responsible for progesterone insensitive tumor growth, we started to identify, isolate and characterize progesterone regulated genes which are differentially expressed between the for growth progesterone sensitive endometrial carcinoma cell line IK-3H12 and the for growth progesterone insensitive endometrial carcinoma cell line ECC-1. In order to do so, a differential display PCR method was used (Chang et al., 1997). With this method several genes were identified which could be involved in progesterone regulated growth inhibition of endometrial carcinoma cells. This insight in molecular mechanisms involved in progesterone insensitive tumor growth may lead to new tools for better recognition and treatment of progesterone insensitive endometrial carcinomas.

Materials and Methods

Materials

The synthetic progesterone Medroxyprogesterone-acetate (MPA; Sigma

Chemical Co., St Louis, USA) was used at 10^{-6}M concentration because this agent is used at comparable serum concentration in the treatment of patients with endometrial cancer. Primers used are from Pharmacia Biotech (Roosendaal, The Netherlands).

Cell Culture

IK-3H12 cell line was a gift from Dr. Masato Nishida (Japan) and represents a well-differentiated human endometrial carcinoma cell line, expressing progesterone receptors. The ECC-1 cell line is a well-differentiated endometrial carcinoma cell line derived from a xenograft of human endometrial carcinoma tissue, expressing progesterone receptors. The ECC-1 cell line was a gift from Dr. P.G. Satyaswaroop (Pennsylvania, USA). The T47D cell line was a gift from Dr. B. van der Burg (Hubrecht Laboratory, Utrecht, The Netherlands). The T47D cell line is a well-differentiated human breast cancer cell line, expressing progesterone receptors. The IK-3H12 and the T47D cell lines were maintained at 37°C in DMEM/F12 culture medium supplemented penicillin/streptomycin, 10% FCS and 10^{-9}M estradiol. The ECC-1 cell line was maintained at 37°C in RPMI 1640 culture medium supplemented with penicillin/streptomycin, 10% FCS and 10^{-9}M estradiol. For the experiments, cells were passaged into indicated media supplemented with 10% stripped FCS (dextran-coated charcoal-treated FCS) replacing non-stripped FCS. Cells were cultured for the indicated times with or without 10^{-6}M MPA.

Progesterone induced growth inhibition

The three cell lines were cultured in the presence or absence of 10^{-6}M MPA for four days. Cells were harvested in 0,1M NaOH and DNA concentration was measured.

Differential display PCR

The three cell lines were cultured for 0, 8 or 48 hours in the presence of 10^{-6}M MPA. Total RNA was extracted by lyzing the cells with 3 M litium chloride/ 6 M urea (Auffray & Rougeon, 1980). Differential display PCR was performed as described by Chang et al., (Chang et al., 1997) In short: total RNA (2μg) was reverse transcribed into cDNA using arbitrary primers. PCR

was performed by amplifying the cDNA with the same or with other arbitrary primers. PCR products were separated on a 6% denaturing polyacrylamid gel in presence of 8 M urea. The gel was dried and exposed to X-ray film. Differentially expressed bands were excised from the gel and stored at -20°C.

Results

The IK-3H12 endometrial carcinoma cell line and the T47D breast cancer cell line showed a reduction in growth in response to exposure to 10^{-6} M MPA. The ECC-1 endometrial carcinoma cell line did not show a growth response to 10^{-6} M MPA (Figure 5.1). The IK-3H12 and T47D cell lines therefore represent for growth progesterone sensitive carcinoma cell lines and the ECC-1 cell line represents a for growth progesterone insensitive cell line.

The aim of the present study is to identify, isolate and characterize progesterone-regulated genes, which are involved in progesterone induced growth inhibition in endometrial carcinoma tissue. A prerequisite for a progesterone regulated gene involved in growth inhibition is that it should be progesterone regulated in the for growth progesterone sensitive endometrial carcinoma

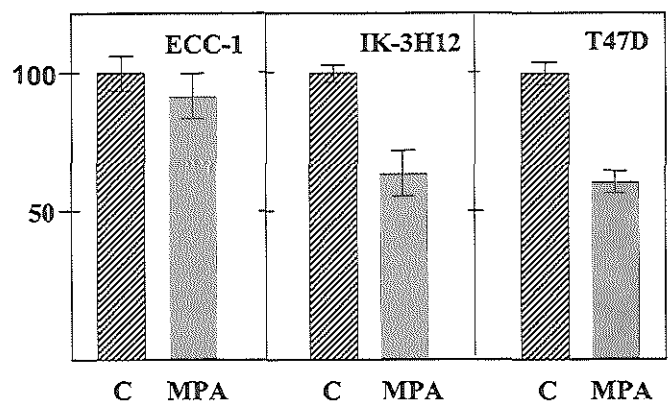


Figure 5.1: Medroxyprogesterone induced growth inhibition. The three indicated cell lines were cultured in the presence (gray column) or absence (shaded column) of 10^{-6}M MPA for four days. Cells were harvested in 0,1M NaOH and DNA concentration was measured and calculated as percentage of control.

Table 5.1. Progesterone regulated cDNA-clones in the ECC-1, IK-3H12 and T47D cell lines

Clone nr	ECC-1	IK-3H12	T47D
4	not regulated	regulated	not regulated
6	not regulated	regulated	not regulated
8	not regulated	regulated	not regulated
31	not regulated	regulated	not regulated
11	regulated	regulated	not regulated
33	regulated	regulated	regulated
34	regulated	regulated	regulated
35	regulated	regulated	regulated

cell line IK-3H12; the gene should not be progesterone regulated in the for growth progesterone insensitive cell line ECC-1.

Eight cDNA clones have been found to be progesterone regulated in the progesterone sensitive endometrial carcinoma cell line IK-3H12 (Table 5.1). Four of these eight progesterone regulated genes were not regulated in the for growth progesterone insensitive endometrial cancer cell line ECC-1 (cDNA clone 4, 6, 8, 31). An example of such a cDNA clone is clone 31, which is shown in figure 5.2. These genes could be potentially important genes, as alterations in the expression or function of these genes could be involved in determining the degree of progesterone induced cell growth inhibition in endometrial carcinoma tissue. To be able to distinguish between early and late responsive genes, the expression of the genes was compared between 8 and 48 hours exposure to 10^{-6} M MPA. Clones 4, 6, 8 and 31 represent early-regulated genes. The other four of the eight progesterone regulated cDNA clones in IK-3H12 (clone 11, 33, 34 and 35) were found to be regulated in both endometrial cancer cell lines (Table 5.1). Furthermore, they appeared to be early regulated in the IK-3H12 and late regulated in the ECC-1.

In order to have some indications that the progesterone regulated genes are or are not endometrial carcinoma specific involved in growth regulation, the expression in the for growth progesterone sensitive human breast cancer cell line T47D was also investigated. None of the four progesterone-regulated genes (cDNA clone 4, 6, 8, 31) appeared to be progesterone regulated in T47D, indicating that the involvement of these genes in progesterone induced growth inhibition is tissue specific. Apart from the eight differentially expressed cDNA clones in the IK-3H12, thirty-seven clones were differen-

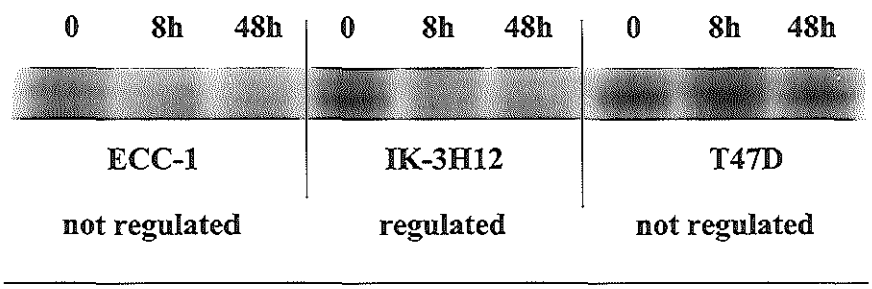


Figure 5.2: cDNA clone 31: a progesterone regulated gene in IK-3H12 and potentially involved in growth inhibition. Indicated cell lines were cultured for 0, 8 and 48 hours in the presence of 10^{-6}M Medroxyprogesterone. mRNA was isolated and reversed transcribed into cDNA using arbitrary primers. The cDNA was amplified with arbitrary primers in presence of ^{33}P-dATP. The products were separated on a sequencing gel and exposed to X-ray film.

tially expressed in either ECC-1 or T47D or both. As these fragments are not differentially expressed in the for growth progesterone sensitive endometrial carcinoma cell line IK-3H12, they do not seem to be involved in growth regulation. These genes could be interesting progesterone regulated genes involved in cellular mechanisms other then growth regulation.

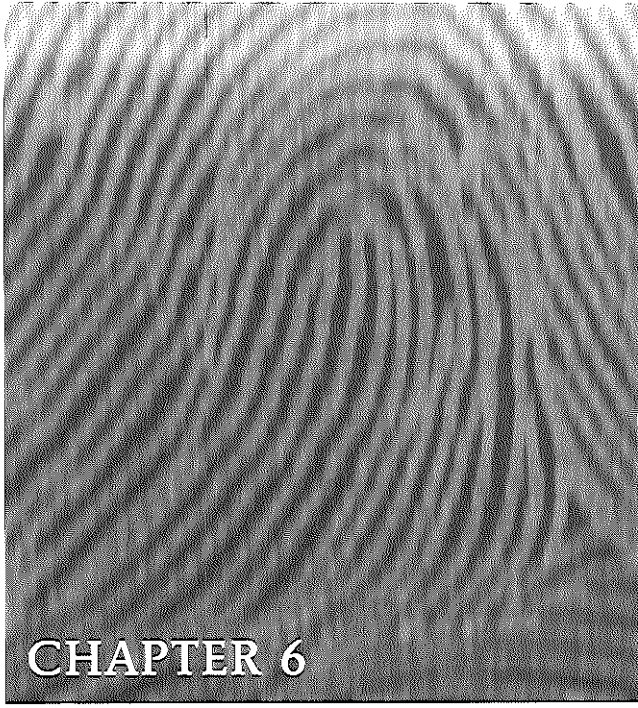
Discussion

As tumor development is thought to be associated with a cascade of genetic alterations, it can be expected that genetic changes are involved in the development of progesterone insensitivity in endometrial carcinomas (Fearon & Vogelstein, 1990; Knudson, 1993). The expression and alterations of some of the known oncogenes and tumor suppressor have been studied in endometrial carcinoma tissue (Berchuck & Boyd, 1995; Esteller et al., 1997; Jones et al., 1997). For example the tumor suppressor gene P53 is found to be mutated in 30% and K-ras, a proto-oncogene, in 20% of the endometrial cancers (Berchuck & Boyd, 1995; Esteller et al., 1997; Jones et al., 1997). Little however is known about progesterone regulation of these genes or the involvement of these genes in progesterone induced growth inhibition. In order to obtain more insight in the molecular mechanism involved in the transition of a

for growth progesterone sensitive endometrial carcinoma into a for growth progesterone insensitive endometrial carcinoma, the present study aimed to identify progesterone regulated genes in endometrial cancer involved in progesterone regulated growth inhibition. With the use of ddPCR eight progesterone regulated genes have been identified in the for growth progesterone sensitive endometrial carcinoma cell line IK-3H12. To be involved in growth inhibition these progesterone regulated genes should not be regulated in the for growth progesterone insensitive endometrial carcinoma cell line ECC-1. Four of these eight progesterone-regulated genes appeared not to be regulated in the ECC-1 cell line. These genes could be the important genes, involved in induction of progesterone regulated growth inhibition. Alterations in the function or expression of these genes could result in the development of insensitivity of endometrial carcinoma tissue to treatment with progesterone. Currently these four progesterone-regulated genes are being cloned and characterized. Identification and characterization of these genes will provide more insight in the molecular mechanism involved in progesterone regulation of growth in endometrial cancer. This insight will help to improve the treatment of women with advanced / recurrent endometrial carcinomas.

Acknowledgements
We are grateful to Dr. Masato Nishida for the gift of the IK-3H12 cell line, to Dr. P.G. Satyaswaroop for the gift of the ECC-1 cell line and Dr. B. van der Burg for the gift of the T47D cell line.

Notes
1. After this paper was published, sequence analysis revealed that three of the four progesterone regulated cDNAs matched with known genes (dsRNA adenosine deaminase, ninein, endothelin converting enzyme-1). During characterization of these genes, it was observed that the IK-3H12 cells lose expression of the human Progesterone Receptor (hPR) rapidly. Further characterization of the genes was therefore postponed. Subsequently, IK-3H12 were stably transfected with hPR in order to create hPR expressing endometrial carcinoma cell lines (see Chapters 6 and 7).
2. The genes described in this paper are not included in the Atlas Human Cancer cDNA array used for the analysis described in Chapters 6 and 7.
3. For a discussion of the observations on differential gene expression, see Chapter 8.



CHAPTER 6

Distinct functional differences of human progesterone receptors A and B on gene expression and growth regulation in two endometrial carcinoma cell lines

Distinct functional differences of human progesterone receptors A and B on gene expression and growth regulation in two endometrial carcinoma cell lines.

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Summary

To study the functional differences between the two progesterone receptor isoforms (hPRA and hPRB) in human endometrial cancer, two new endometrial carcinoma cell lines were created; one expressing hPRA and one expressing hPRB. A well differentiated hPR-negative Ishikawa cell line was stably transfected with either hPRA or hPRB cDNA. Transfected cells were selected and two cell lines expressing approximately equal amounts of receptor were isolated; one expressing hPRA (PRA-14) and one expressing hPRB (PRB-59). Cell growth experiments revealed a growth inhibitory response to progestins (MPA and R5020) in the PRB-59 cells, but not in the PRA-14 cells. Differences in expression of genes targeted by the two isoforms were studied using a cDNA expression array technique. A different set of genes appeared to be progesterone regulated in the PRA-14 cells compared to the PRB-59 cells. None of the genes were regulated by both hPRA and hPRB. IGFBP3 expression was studied in more detail as an example of a gene regulated in PRB-59 cells and not in PRA-14 cells. In conclusion, a new model to study functional differences between the two hPR isoforms in human endometrial carcinoma cells has been established. This model revealed distinctive differences in target gene regulation between the two hPR isoforms. Moreover, antiproliferative actions of progesterone on human endometrial cancer cells could only be observed in the PRB expressing cell line.

Introduction

The ovarian hormone progesterone plays an important role in controlling uterine homeostasis. Progesterone initiates transformation of uterine stromal cells to decidual cells in case of pregnancy (Curtis et al., 1999) and, moreover, is involved in a complex cross talk with estradiol in order to control proliferation and differentiation of endometrial epithelium during the menstrual cycle. Estrogens stimulate proliferation of the endometrial epithelium (Quarmby & Korach, 1984), initiate an inflammatory response in the stromal part of the endometrium and induce uterine hyperemia (De & Wood, 1990). Progesterone has been found to inhibit these estrogen induced changes of the endometrial epithelium and stimulates glandular and stromal differentiation (Martin et al., 1973). These observations have been confirmed in the progesterone receptor (PR) knockout mice model showing unopposed estrogen-dependent endometrial epithelium hyperplasia, stromal hypocellularity, and uterine inflammation (Lydon et al., 1996; Lydon et al., 1995). Moreover, in clinical settings progesterone is effectively used as treatment to reverse endometrial hyperplasia and to decrease the growth of endometrial tumors (Ehrlich et al., 1981).

Progesterone mediates its effects through its nuclear receptor. The human progesterone receptor (hPR) belongs to the super family of nuclear receptors, which includes steroid, thyroid hormone, vitamin D, and retinoic acid receptors (Evans, 1988). The hPR exists as two isoforms: hPRA (94 kDa) and hPRB (114 kDa) (Horwitz & Alexander, 1983). The hPRA is a truncated form of the hPRB lacking the first 164 NH_2-terminal amino acids. Both forms have similar DNA and ligand binding affinities (Lessey et al., 1983). However, hPRA and hPRB are not functionally identical. Their relative efficiencies to activate target genes appear to vary according to the promoter and cell context (Meyer et al., 1989). Moreover, hPRA has been shown to act as a strong trans-dominant repressor of hPRB mediated transcription in specific promoter and cell contexts (Vegeto et al., 1993).

The two isoforms originate from the same hPR gene by transcription regulation by two distinct promoters respectively (Kastner et al., 1990). In human target cells both isoforms are coexpressed, generally at the same level (Graham & Clarke, 1997). However, the promoters are regulated independently, which can give rise to varying hPRA: hPRB ratio in target tissues under certain physiological circumstances (Kastner et al., 1990; Kraus et al., 1997).

In the endometrium, the hPRA predominates in stromal cells throughout the menstrual cycle, whereas in epithelial cells, a shift occurs during the early secretory phase from hPRA to hPRB (Mote et al., 1999). The varying ratio of the two isoforms among different target cells and different physiological circumstances suggests that the differential expression level of PRA and PRB may determine the cellular response to progesterone. Using PRA knockout (PRAKO) mice, Mulac-Jericevic et al. (Mulac-Jericevic et al., 2000) showed that progesterone treatment of these animals resulted in PRB specific gene regulation and PRB dependent increase in proliferation of endometrial cells. These results indicate that imbalance in the expression and/or activities of the two isoforms can have important consequences for normal endometrial development and may play a role in the development of endometrial cancer. The suggestion that an imbalance in the hPRA: hPRB ratio can be involved in carcinogenesis is supported by the observation that in transgenic mice overexpressing PRA, the mammary gland exhibits ductal hyperplasia with a disorganized basement membrane and decreased cell-cell adhesion; features commonly associated with neoplasia (Shyamala et al., 1998). Moreover, in *in vivo* studies high levels of hPRA were observed in a subset of breast tumors (Graham et al., 1995), and *in vitro* studies have suggested that poorly differentiated endometrial cancers express only hPRA (Kumar et al., 1998).

To gain more insight in the physiological implications of changing PRA: PRB ratios during carcinogenesis, understanding of the distinct effects of PRA and PRB on cellular function is essential. So far, insights into the differences between PRA and PRB transcriptional activity, have been obtained by transient cotransfection of PRA and/or PRB with reporter constructs or by knocking out PRA or both PR isoforms in transgenic mice. Furthermore, most of these studies have been carried out in cells that are normally not a target cell for progesterone (Kraus et al., 1997; Meyer et al., 1989; Tora et al., 1988; Vegeto et al., 1993). To study the distinct function of hPRA and hPRB on cellular processes in endometrial cancer cells, we stably transfected hPRA and hPRB cDNA plasmids into (PR-negative) Ishikawa endometrial carcinoma cells. The effects of progesterone on cell growth and gene expression have been studied in these hPRA and hPRB expressing endometrial cancer cell lines.

Materials and Methods

Recombinant Plasmids

The hPR-cDNA cloned into the pSG5 expression vector was a generous gift from Dr. E. Milgrom (Kremlin-Bicetre, Paris, France). This vector was used to create hPRA and hPRB expression plasmids, respectively. A 3063 bp cDNA fragment encoding hPRB was isolated from pSG5-hPR by excision of the *Eco*R1-*Xba*1 fragment (the *Eco*R1 site being artificially introduced at position +14 upstream from the first AUG codon). A 2982 bp cDNA fragment encoding hPRA was isolated by excision of the *Bam*H1-*Xba*1 fragment (the *Bam*H1 site being a hPR restriction site at position -68 downstream from the first AUG codon and +424 upstream from the second AUG codon). Subsequently, the hPRA and hPRB cDNA fragments were inserted into the pcDNA3.1 expression vector (Invitrogen Corporation, Carlsbad, CA), containing a cytomegalovirus (CMV) promoter and a neomycin selection marker. Sequence analysis of the created plasmids was performed to verify proper ligation of the hPR cDNA fragments into the vector.

Cell culture

Ishikawa cells are derived from a well-differentiated human endometrial adenocarcinoma (Nishida et al., 1996) and were a generous gift from Dr. Masato Nishida (Tsukuba, Japan). The T47D cells are derived from well-differentiated human breast cancer and were a generous gift from Dr. B. van der Burg (Utrecht, The Netherlands). Both cell lines were negative for mycoplasma contamination, determined using Mycoplasma-Plus-PCR-Primer-Set (Stratagene, La Jolla, CA). All cells were maintained in DMEM/F12 (Dulbecco's modified Eagle's medium/Ham's F12 (1:1 mix) with 15mM Hepes, with L-glutamine) supplemented with penicillin/streptomycin; in the presence of 7.5% fetal calf serum and 10^{-9} M estradiol in a 37°C incubator with 5% CO_2. The cells transfected with either hPRA or hPRB were cultured under continuous selection pressure with neomycin (G418: 500 µg/ml, Invitrogen Corporation, Carlsbad). For total RNA isolation (northern blots and microarrays), cells were cultured for the indicated time in the presence or absence of 10^{-6} M Medroxy Progesterone Acetate (MPA, Sigma Chemical co., St Louis, MO) in DMEM/F12 supplemented with 7.5% dextran-coated charcoal-treated fetal

calf serum, 10^{-9} M estradiol and, for the transfected cells, neomycin at 500 µg/ml. Total RNA was isolated by lyzing the cells with 3M Lithium chloride/ 6M Urea as described by Auffray and Rougeon (Auffray & Rougeon, 1980). The RNA was purified as described by Blok et al (Blok et al., 1995). For the growth studies the cells were cultured in DMEM/F12 supplemented with 7.5% dextran-coated charcoal-treated fetal calf serum, 10^{-9} M estradiol and with different concentrations of Medroxy Progesterone Acetate (MPA, Sigma Chemical Co., St Louis, USA) or R5020 (NEN Life Science Products, INC, Boston, MA) as is indicated in the legends to the figures.

Construction of two Ishikawa cell lines stably expressing hPRA and hPRB, respectively

Ishikawa cells were plated into 40 cm^2 culture dishes and cultured to 50% confluency. The hPRA-pcDNA3.1+ plasmid, the hPRB-pcDNA3.1+ plasmid and the control "empty-"pcDNA3.1+ vector (Invitrogen Corporation, Carlsbad, CA) were transfected into the Ishikawa cells using FuGENE™ 6 Transfection Reagent (Roche Diagnostics Co. Indianapolis, IN) following instructions provided by the manufacturer. The cells were cultured in the presence of the selection marker neomycin (500 µg/ml) and cloned by limited dilution. Surviving clones were selected and transferred to 24-well plates. Thirty Ishikawa-hPRA clones, seventy Ishikawa-hPRB clones and seventeen Ishikawa-pcDNA3.1+ clones were analyzed for hPR expression by a ^{3}H-R1881-binding assay. A hPRA clone (PRA-14) and a hPRB clone (PRB-59), showing high specific binding to ^{3}H-R1881, were selected and further characterized.

^{3}H-R1881-binding assay

The cells were cultured in 6-well plates, under standard conditions as described above, to 75% confluency. The cells were incubated with 8nM ^{3}H-R1881 (NEN Life Science Products, Inc, Boston, MA) in presence or absence of 4µM non-labeled R1881 (NEN Life Science Products, Inc, Boston, MA) for 2 h in a 37°C incubator. The cells were washed five times with phosphate buffered saline (PBS) at 0°C. The cells were lysed in 1M NaOH for 30 min at 60°C. Radioactivity was measured in a liquid scintillation counter and protein concentrations were measured using Bradford's reagents.

Hormone binding assay (Scatchard analysis)

Receptor binding parameters were determined as described under R1881-binding assay, with the exception that different amounts of ^{3}H-R1881 (16 nM, 8nM, 4 nM, 2 nM, 1 nM, 0.5 nM, 0.25 nM, 0.125 nM, 0.06 nM, 0.03 nM, 0.015 nM, 0.008 nM) were used in the presence or absence of 2000-fold unlabelled R1881. Scatchard plots were constructed using Excel software (Microsoft) and Kd and B_{max} values were calculated.

Northern-blotting

For total RNA isolation, cells were cultured for 48 hours in the presence or absence of 10^{-6} M Medroxy Progesterone Acetate (MPA, Sigma Chemical co., St Louis, MO) in DMEM/F12 supplemented with 7.5% dextran-coated charcoal-treated fetal calf serum, 10^{-9} M estradiol and, for the transfected cells, neomycin at 500 µg/ml. Total RNA was isolated by lyzing the cells with 3M Lithium chloride/ 6M Urea as described by Auffray and Rougeon (Auffray & Rougeon, 1980). The RNA was purified as described by Blok et al (Blok et al., 1995). Northern blotting was performed as described by Chang et al (Chang et al., 1997). As a probe, to detect IGFBP3 mRNA on the northern blot, a 2.4 kb EcoR1 cDNA fragment containing the complete mouse IGFBP3 coding sequence was used. This cDNA was kindly provided by Dr. S.L.S. Drop (Department of Pediatrics, division of Endocrinology,Erasmus Medical Center Rotterdam, The Netherlands). To detect PR mRNA on the northern blot, a 0.5 kb *Bgl*1 cDNA fragment was used, containing coding sequence present in both the hPRA and the hPRB. The complete hPR cDNA was a generous gift from Dr. E. Milgrom (Kremlin-Bicetre, Paris, France).

Western immuno-blotting

The cells were cultured as described before to 75% confluency. The cells were washed twice with PBS, lysed in RIPA buffer (40mM Tris-HCL (pH 7.4), 5mM EDTA (pH 8.0), 10% glycerol, 10 mM Sodiumphosphate, 10 mM Sodiummolybdate, 50 mM Sodiumfluoride and 0.5 mM Sodiumorthovanadate 10mM DTT, 1% Triton, 0.08% SDS, 0.5% Deoxycholate, and protease inhibitors: 6 mM PMSF, 5 mM Bacitracin, 5 mM Leupeptin) and centrifuged for 10 min at 350.000 g, 4°C. The proteins were separated on a SDS polyacrylamid gel and transferred to nitrocellulose (Schleicher & Schuell GmbH, Dassel, Germany). The PR (C-20) rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with the membrane as follows: The

membrane was rinsed with PBS-tween and blocked for 1 h with blocking solution. The PR (C-20) antibody was diluted 1: 2000 in blocking solution and incubated with the membrane for 1 h. The membrane was washed four times 15 min with PBS-tween. Antibody-peroxidase conjugate was diluted 1: 2000 in blocking solution and incubated with the membrane for 1 h. The membrane was washed four times for 15 min with PBS-tween. The PR bands were detected by using Luminol chemiluminescence procedure (NEN Life Science Products, INC, Boston, MA) and visualized by exposing the blot to X-ray film (Kodak X-Omat, New Haven, CT) for at least 1 min.

Cell Growth Experiments

The cells were seeded at 20,000 cells per well in 24-well plates and cultured for the indicated time in the presence or absence of the indicated concentration of Medroxy Progesterone Acetate (MPA, Sigma Chemical Co., St Louis, USA) or R5020 (NEN Life Science Products, INC, Boston, MA) in DMEM/F12 supplemented with 7.5% dextran-coated charcoal-treated fetal calf serum, 10^{-9} M estradiol and, for the transfected cells, neomycin at 500 µg/ml. The cells were washed twice in PBS (10 min.), lysed in 150 µl 1M NaOH for 30 min at 60°C, the lysates were further diluted 1:9 in H_2O and growth differences were obtained by measuring OD 260 values of the lysates.

Statistical analysis

The experiments described in Figure 6.3B were performed three times and paired sample t-tests were performed using the SPSS programme. Differences between control and treatments were considered significant when $p < 0.01$.

Transcriptional activity

Atlas Human Cancer cDNA expression array. The Atlas Human Cancer cDNA expression array (7742-1) was purchased from Clontech Laboratories, Inc. (Palo Alto, California, USA). Two identical nucleic acid arrays were supplied containing 588 cancer related human cDNA's spotted as duplicates on nylon membranes. A list of the genes spotted on the array, including array coordinates, is available at Clontech's web site http://www.clontech.com. Total RNA was reverse transcribed into cDNA using a mixture of array gene-specific primers and was labeled with ^{32}P-dATP. Probe purification and hybridization to the array was performed following the array's user manual. The array was exposed to a phosphor-imaging screen at room temperature for 24 hours

and scanned using the Phosphor Imager (Molecular Dynamics, Sunnyvale). The results were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale). A grid was applied to the image of the blot to quantify the intensity of hybridization of every spot. According to the array manufacturer (Clontech Laboratories, Palo Alto, CA), the radioactive cDNA signal is linear for RNA's present at levels of 0.01-3% of the total RNA population.

Numerical analysis of the cDNA expression array data

Numerical analysis of the cDNA expression array data was performed as described previously (Smid-Koopman et al., 2000). In short: The expression data obtained from Phosphor Imager analysis were logarithmic (^{10}log) transformed. Hybridization of one cDNA pool to two twin array membranes confirmed that gene expression profiles, obtained using this cDNA array technique, are highly reproducible (Smid-Koopman et al., 2000). Using *xy-scatter plot* the expression levels of the genes for two experiments can be compared without the need for preceding normalization of the data. The *least-squares regression line* y=ax+b is the line with the smallest sum of squared vertical distances between the points of the xy-scatter plot and the line. In the least-squares regression line ($y=\boldsymbol{a}x+\boldsymbol{b}$) the slope value $\boldsymbol{a}$ represent the differences in hybridization intensity and the intercept value $\boldsymbol{b}$ represent the differences in background signal. *R-squared value* is the Pearson correlation coefficient of the least-squares regression line and can be considered as a quantification of similarity between compared data.

The *perpendicular-line distance* $\boldsymbol{d}$ $\{\boldsymbol{d}=[1/\sqrt{(1+a^2)}]\cdot[y-(ax+b)]\}$ of every point to the least-squares regression line represents the difference in expression level of the specific gene for the two conditions compared. Using percentile ranking of the distances $\boldsymbol{d}$, the genes located outside the ninety-seven percentile were defined as differentially expressed. 10^d represents the order of *magnitude* of the differential regulation of the gene.

Using the 97% interval to identify differentially expressed genes will always result in a fixed amount of differential expressed genes, regardless of the order of magnitude of the regulation. It seems preferable to select genes only after weighing the degree of regulation. The order of magnitude at which the regulation of gene expression has functional biological effects however, has yet to be established. Hybridization of one cDNA pool to two twin array membranes showed that variation within one experiment could be as much

as 1.5 fold (Smid-Koopman et al., 2000). Therefore, genes with expression difference less than 2.0 fold were not considered to be regulated genes.

Results

Establishment of two Ishikawa sub-cell lines: PRB-59 and PRA-14

To study the distinct functions of the two hPR isoforms, hPR-negative Ishikawa endometrial cancer cells were stably transfected with hPRA or hPRB expression plasmids. Using northern and western blot analysis (Figure 6.1) the hPR-negative status of the parental Ishikawa cells was established. Furthermore, using a ^{3}H-R1881-binding assay, significant specific binding could not be detected, indicating the absence of progesterone receptors in the parental cells. In order to measure potential binding of the ^{3}H-R1881 to the androgen receptor (Zava et al., 1979), a 500 fold molar access triamcinolone acetonide was used to block any progesterone receptors. No specific binding above background could be detected, indicating that the parental Ishikawa cell line do not express androgen receptors. Thirty hPRA transfected clones, seventy hPRB transfected clones and seventeen empty vector transfected clones were analyzed for hPR expression with a ^{3}H-R1881-binding assay. One hPRA clone (PRA-14) and one hPRB clone (PRB-59), both showing the highest specific binding of ^{3}H-R1881, were selected for further characterization. The empty vector clones and the parental Ishikawa cells (IK-par) showed no specific 3H-R1881 binding. The hPR expression of the two sub-cell lines (PRA-14, PRB-59) was verified on northern and western blot, and T47D breast cancer cells expressing both hPRA and hPRB were used as a positive control (Figure 6.1). Both the PRA-14 and the PRB-59 sub-cell line were positive for PR-mRNA and protein (Figure 6.1). The relatively poor resolution of the northern blot prevents detection of a difference in transcript length between the PRA and PRB transcripts (the PRB transcript is 81 bp longer than the PRA transcript).

Using Scatchard analysis a Kd value of: 0.1 ± 0.1 nM, B_{max} 0.05 ± 0.003 nM and 12.0 pmoles/mg protein: equals 22000 sites per cell for the hPRA was established in the PRA-14 cells. In the PRB-59 cells a Kd value of: 0.2 ± 0.2 nM, B_{max} 0.08 ± 0.003 nM and 12.7 pmoles/mg protein: equals 23500 sites per cell was established for the hPRB (Figure 6.2). These Kd values are

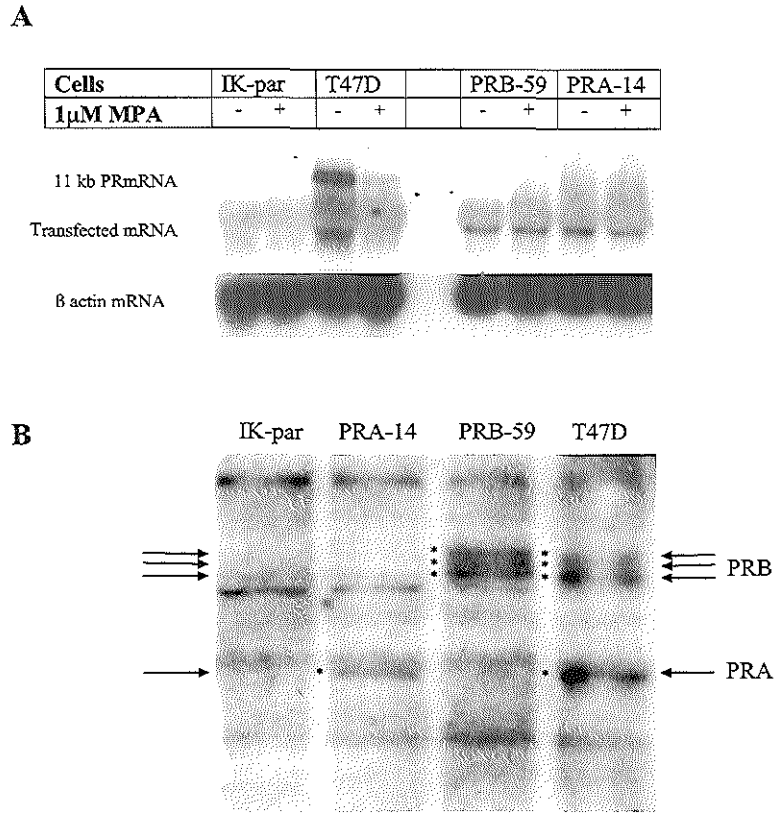


Figure 6.1: Expression hPRA and hPRB mRNA (A) and protein (B) in the cell lines. The RNA expression pattern of the transfected human progesterone receptors was verified on northern blot (A) and on western blot (B) for both Ishikawa sub-cell lines: PRA-14 and PRB-59. The parental Ishikawa cells (IK-par) and the T47D breast cancer cells were used as negative and positive controls, respectively. Before RNA isolation, the cells were cultured for 48 hours in the presence (+) or absence (-) of 1 μM MPA. The T47D cells express endogenous hPR (11 kb PR mRNA). The transgenes both run at 3kb (transfected mRNA). Equal amounts of RNA were loaded on the northern blot as indicated by ethidium bromide staining of total RNA (not shown). Hybridization to β-actin mRNA indicates that equal amounts of mRNA were loaded for each cell line cultured either in the presence or absence of MPA (1 μM). For the western blot (B) equal amounts of protein lysate were loaded to the gel for the IK-par, PRA-14 and PRB59 cells. For T47D cells 4-fold less protein was loaded because the PR-protein expression is approximately 5-fold higher in this cell line compared to the PRA-14 and PRB-59 cells. PRB and PRA protein bands are indicated by arrows in the left and right margin and by asterixes in the figure.

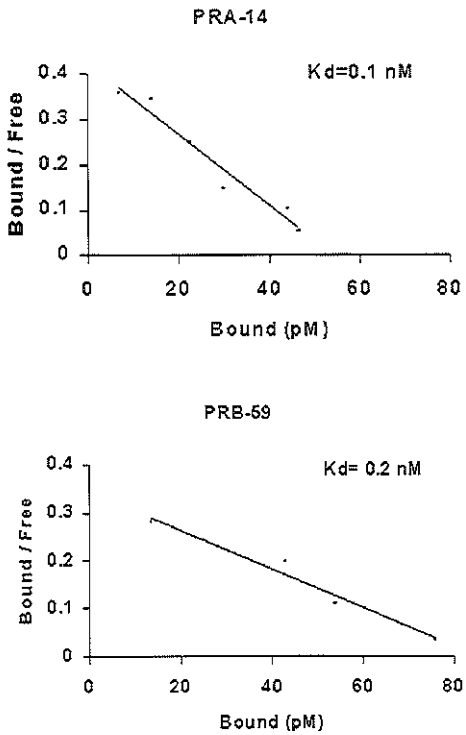


Figure 6.2: Scatchard analysis of the hPRA and hPRB expressing Ishikawa sub-lines: PRA-14 and PRB-59. Cells were incubated with different amounts of ^{3}H-R1881 (16 nM, 8nM, 4 nM, 2 nM, 1 nM, 0.5 nM, 0.25 nM, 0.125 nM, 0.06 nM, 0.03 nM, 0.015 nM, 0.008 nM) in presence or absence of 2000 fold non-labeled R1881. Scatchard plots were constructed using Excel software.

within the range of published hPR-Kd-values for endometrial cells (De Goeij et al., 1988; Satyaswaroop et al., 1982).

Progestins inhibit growth of Ishikawa cells expressing hPRB

Progesterone is known to inhibit estrogen-induced proliferation of endometrial epithelial cells (Martin et al., 1973). Whether PR can also have an estrogen independent effect on cell proliferation in endometrium cells is unknown. Moreover, whether the PR effects are generated via PRA, via PRB or via both isoforms is also unknown. To determine the distinct effects of the two liganded hPR isoforms on growth regulation of endometrial epithelial cells, the PRA-14 and PRB-59 cell lines were cultured in the presence of different progestins, e.g. Medroxy Progesterone Acetate (MPA) and R5020, in varying concentrations. MPA was used because this compound is applied,

at high concentrations (1μM), for treatment of advanced and/or recurrent endometrial cancer in clinical practice.

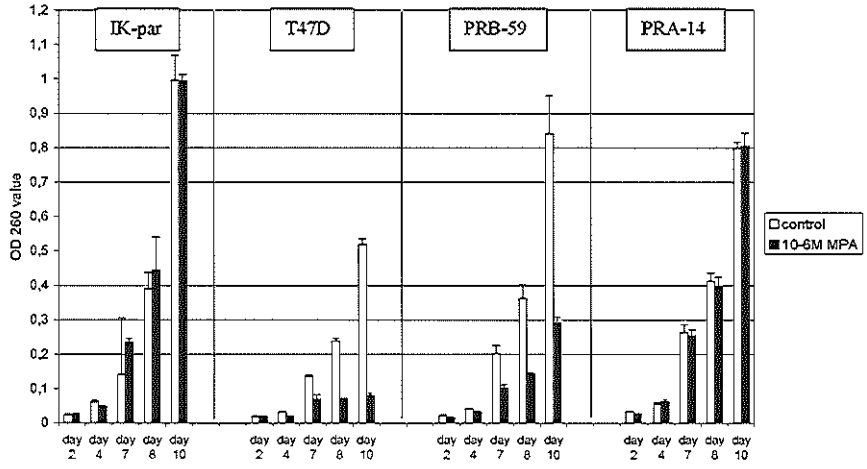
It was observed that 1 μM MPA effectively inhibited estrogen-induced growth of T47D cells (Figure 6.3 A and 6.3 B). Furthermore, 1 μM MPA could also inhibit growth of PRB-59 cells irrespective of the presence or absence of estrogen in the culture medium (Figure 6.3 B). Interestingly, neither the parental Ishikawa cells nor the PRA-14 cells showed growth inhibition during culture in the presence of progestins (Figure 6.3 A and 6.3 B). A concentration curve of PRB-59 cells cultured in the presence of different amounts of MPA for 10 days showed an ED_{50} of between 0.01nM and 0.1 nM (Figure 6.3 C), indicating that also at low MPA concentrations growth inhibition is observed. As expected, when PRB-59 cells were cultured in the presence of 1 nM MPA in combination with 0.1 μM RU486 (anti-progestin) the progesterone-induced growth inhibition became impaired (Figure 6.3 D).

Distinct differences in gene expression regulation between PRA and PRB

In order to determine the effects of the two PR isoforms on physiological gene expression in endometrial cancer cells, gene expression profiles of the PRA-14 and PRB-59 endometrial cancer cell lines were compared. The cells were cultured with or without 1 μM MPA for 48 hours. The 48 h time point was chosen on the basis of a study in T47D cells showing marked expression differences at this time-point (Kester et al., 1997). Relatively high concentrations of MPA were used (1μM), as this resembles the concentration of MPA used for treatment of advanced and/or recurrent endometrial cancer in clinical practice. Gene expression profiles were generated, using the Atlas human cancer cDNA expression array (Clontech Laboratories, Inc., Palo Alto, California, USA) containing cDNA's of 588 cancer related genes. Typical expression patterns for the cells under the different conditions are shown in Figure 6.4. Progesterone regulated genes were identified using XY-scatter plots (Smid-Koopman et al., 2000).

It was observed that gene expression profiles of the PRA-14 and PRB-59 cells resembled each other fairly well (Figure 6.4). Furthermore, there is also a high degree of similarity between the current arrays and earlier arrays performed on parental Ishikawa cell RNA (Smid-Koopman et al., 2000), implying small genetic differences between the different cell clones. To establish

A


IK-par
T47D
PRB-59
PRA-14
OD 260 value
control
10-6M MPA
day 2
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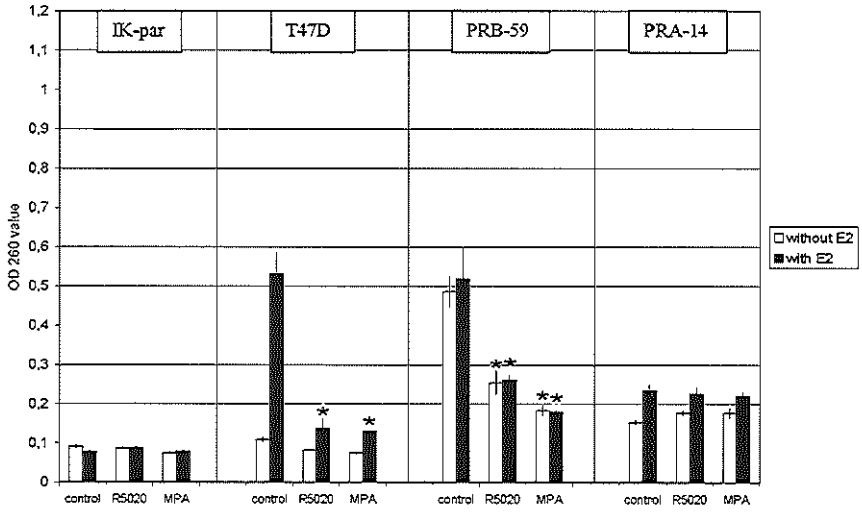
B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PRA-14
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without E2
with E2
control
R5020
MPA

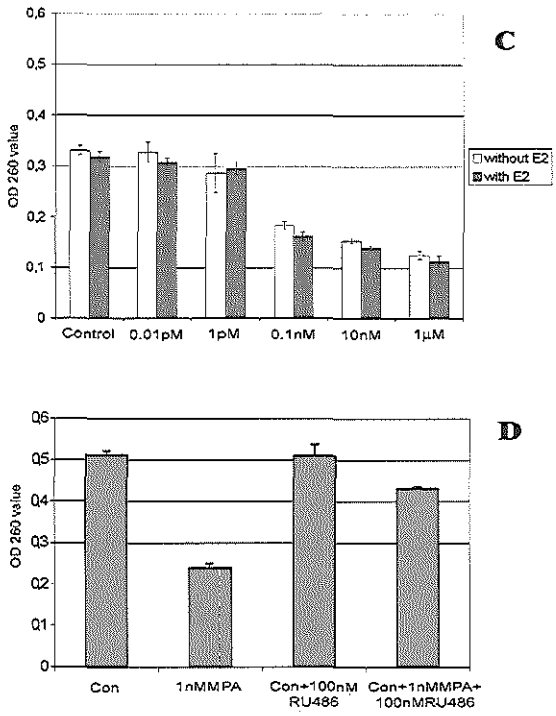


Figure 6.3: Growth characteristics of parental Ishikawa cells, T47D cells, PRA-14 and PRB-59 cells cultured in the presence or absence of progestins, estradiol and/or RU486. Cell growth was measured after different culture periods (2, 4, 7, 8 and 10 days) in the presence (shaded bars) or absence (open bars) of Medroxy Progesterone Acetate (1 µM MPA). This figure represents a single experiment were each point in the curve represents the mean + SD measurements of four wells (A). Cells were grown for six days in medium containing MPA (1µM) in the presence (shaded bars) or absence (open bars) of estradiol (1 nM), after which cell growth was measured. These experiments were performed three times and paired sample t-tests were performed. Differences between control and treatments were considered significant (*) when $p < 0.01$. Each bar represents the average of three different experiments + SD (B). Growth was measured of PRB-59 cells cultured for 6 days in the presence of different concentrations of MPA (0,01 pM, 1 pM, 0,1 nM, 10 nM, 1 µM, combined with (shaded bars) or without (open bars) 1 nM estradiol. This figure represents a single experiment were each point in the curve represents the mean + SD measurements of four wells (C). In order to verify involvement of the progesterone receptor, PRB-59 cells were cultured in the presence or absence of MPA (1 nM) in combination with or without RU486 (100nM). This figure represents a single experiment where each point in the curve represents the mean + SD measurements of four wells (D). The error bars in each figure represent the standard deviations.

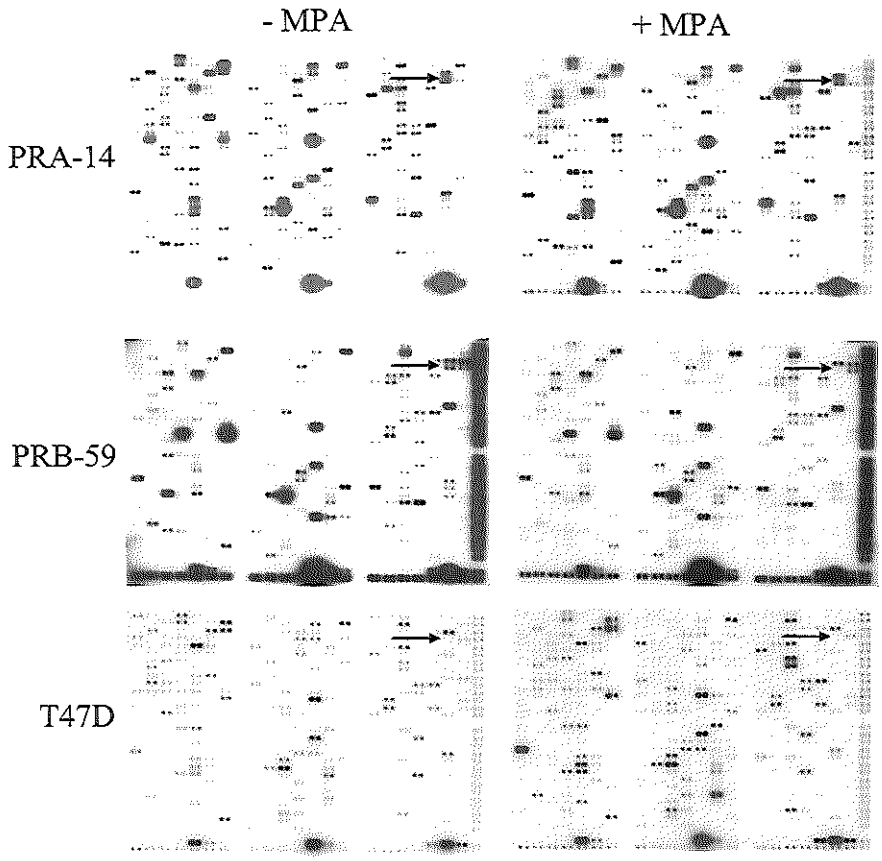


Figure 6.4: Progesterone regulated genes in PRA-14, PRB-59 and T47D cells. Atlas Human Cancer cDNA Expression Array (Clontech, USA) was hybridized with ^{32}P-labeled cDNA probes obtained from total RNA of the PRA-14, PRB-59 and T47D cells. All cell lines were cultured in the absence (-MPA) or presence (+MPA) of 1 μM MPA. The array contains 588 cancer related human cDNAs spotted as duplicates. A complete list of the names of the cDNA's spotted on the array is available at CLONTECH's web site (http:\\www.atlas.clontech.com.).

the potential of the array technique for identification of progesterone regulated genes, T47D breast cancer cells were used as positive controls (Figure 6.4). The T47D cells are known to express progesterone target genes (Kester et al., 1997). Using differential display technique, Kester et al., (Kester et al., 1997) identified ten progesterone-regulated genes in the T47D cells. Three

Table 6.1: Genes differential regulated by MPA in T47D, PRA-14 and PRB-59 cell lines, respectively.

	T47D	Fold	PRA-14	Fold	PRB-59	Fold
UP-REGULATED	CD59	3.7	Retinoic acid receptor gamma	2.2		
	Desmoplakin 1	3.1	Integrin beta 4	2.0		
	Notch 2	2.6	MAP kinase P97	2.0		
	Notch group protein (N)	2.6	MTS 1; p16-INK 4	2.0		
	Cytokeratin 19	2.2				
DOWN-REGULATED	Cyclin; PCNA	2.0	Cytokeratin 8	3.3	IGFBP3	3.4
			Cyclin D1; BCL-1 oncogene	2.0	Replication protein A	2.0

Results are based on the XY scatter plot and percentile ranking of the perpendicular-line distance ***d*** of every gene to the least-squares regression line. The genes regulated by a factor 2 or more are shown in this table. The order of magnitude of the regulation of the gene on the array are indicated.

of these genes (CD9, CD59, desmoplakin) are spotted on the Atlas human cancer cDNA expression array and were also identified to be progesterone regulated with the currently used array technique.

The gene expression profiles of the PRA-14 and PRB-59 endometrial cancer cell lines, cultured for 48 hours in presence or absence of 1 μM MPA, were compared using XY-scatter plots (for details see Materials and Methods). On the basis of manufacturers protocol, a 2.0-fold up- or down-regulation was considered to be significant. A different set of genes appeared to be progesterone regulated in the PRA-14 cells as compared to the PRB-59 cells (Table 6.1). Five genes are up-regulated by progesterone in PRA-14 (retinoic acid receptor gamma, integrin beta 4, MAP-kinase p^{97}, p16-INK4 and integrin alpha 7B) and none in PRB-59 cells. Three genes were down-regulated in the PRB-59 cells (IGFBP3, fibronectin and replication protein A), and two in the PRA-14 cells (cytokeratin 8 and cyclin D1). None of the genes were regulated by both hPRA and hPRB. The parental Ishikawa cells and one empty vector clone were used as negative controls. No progesterone-regulated genes could be identified in these cells (data not shown). These observations indicate distinct differences in transcriptional activity between the two hPR isoforms.

IGFBP3 expression was markedly down-regulated in the PRB-59 cells and showed different expression level in PRB-59 cells as compared to PRA-14 and T47D cells (Figure 6.4, indicated by the arrows). To confirm the re-

sults obtained using the cDNA array technique, the expression of this gene (IGFBP3) was studied on northern blot. IGFBP3 cDNA was used as a probe on a northern blot containing total RNA isolated from parental Ishikawa (IK-par), PRB-59, PRA-14 and T47D cells cultured for 48 hours in the presence or absence of 1 μM MPA. The same expression pattern of IGFBP3 was observed on the northern blot as on the cDNA expression arrays: IGFBP3 is regulated by MPA in PRB-59 cells, and not regulated by MPA in PRA-14 and parental Ishikawa cells (IK-par). In T47D cells IGFBP3 expression appears to be absent (Figure 6.5).

Cells	IK-par	T47D		PRB-59	PRA-14
1μM MPA	- +	- +		- +	- +
Relative signal	100 / 120	nd / nd		104 / 20	220 / 260

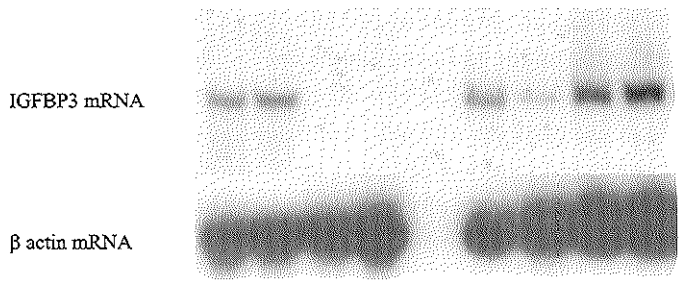


Figure 6.5: IGFBP3 mRNA expression in parental Ishikawa (IK-par), T47D, PRA-14 and PRB-59 cells. The RNA expression pattern of IGFBP3, as observed in figure 4, was verified on northern blot for both Ishikawa sub-cell lines: PRA-14 and PRB-59. The parental Ishikawa cells (IK-par) and the T47D breast cancer cells were used as controls. The radioactive signal was measured using a phosphor-imager and indicated as a signal relative to RNA isolated from IK-par cells cultured in the presence of MPA (this amount is given as 100). IGFBP3 expression in T47D cells could not be detected (nd). Equal amounts of RNA were loaded on the blot as indicated by ethidium bromide staining of total RNA (not shown). Hybridization to β-actin mRNA indicates that equal amounts of mRNA were loaded for each cell line cultured either in the presence or absence of MPA.

Discussion

In a recent paper, Dai et al. (Dai et al., 2001) report on the re-establishment of progesterone control of endometrial cancer cell proliferation using an adenoviral infection system. These authors could show that re-introduction of very high levels of either PRA or PRB into two poorly differentiated endometrial carcinoma cell lines, HEC50 and KLE, restores progesterone induced inhibition of anchorage independent growth. They were also able to show a marked difference between growth of the PRA-expressing cells and the PRB-expressing cells: progesterone induced inhibition of anchorage independent growth was about 50% in PRA-expressing cell lines, while growth was completely abolished in the PRB-expressing cells. As in the current investigations, the level of PRA versus PRB expression in the different cell lines was comparable (Dai et al., 2001). The fact that Dai et al. (Dai et al., 2001), in contrast to the current investigation, could show some growth inhibition in PRA-expressing cell lines can be explained by differences in expression (adenoviral systems usually reach a high level of transgene expression) or by the difference in measured end-parameter (anchorage independent growth versus growth on a culture disk in the present investigations).

The implications of the functional differences between the two hPR isoforms for carcinogenesis of endometrial tissue are currently not very clear. There is one report in literature, using RT-PCR and based on very low number of patients, which seems to point to a more predominant appearance of PRB in more advanced endometrial tumors (Fujimoto et al., 1995); a second report indicated that both PRA and/or PRB may be lost in advanced endometrial cancer (Arnett-Mansfield et al., 2001); and a third *in vitro* study has suggested that poorly differentiated endometrial carcinoma cell lines only express hPRA (Kumar et al., 1998).

Recently, Mulac-Jericevic et al. (Mulac-Jericevic et al., 2000) performed a study in which they have investigated the progesterone response of the endometrium in PRA knockout animals. The authors observed progesterone-induced proliferation of the mouse endometrium, which could only be accounted for by the PRB. The current investigations, however, seem to contradict these observations; showing that progesterone induces an estrogen-independent growth inhibition of the PRB-59 endometrial cancer cells, and therefore indicating growth inhibitory properties of PRB. It is clear that there is discrepancy between the outcome of the different studies, which may

be explained by the large differences between the above mentioned studies: human (Kumar et al., 1998)(current study) *vs.* mouse (Mulac-Jericevic et al., 2000; Shyamala et al., 1998), *in vivo* (Mulac-Jericevic et al., 2000; Shyamala et al., 1998), *vs. in vitro* (Kumar et al., 1998)(current study) and normal growth (Mulac-Jericevic et al., 2000; Shyamala et al., 1998), vs. carcinogenic growth (Kumar et al., 1998)(current study) . Furthermore, in the absence of PRA during the development of the reproductive tract, compensatory changes may have occurred resulting in an enhancement of the proliferative effects of PRB. Another point of consideration is the fact that the currently used cell lines do not express estrogen receptors while *in vivo* these receptors do play an important role in progesterone signaling. These observations indicate that more research into growth inhibiting and -stimulating actions of liganded PRA and PRB is needed.

In the current study only the cells expressing PRB (PRB-59) were found to be growth responsive to progesterone. In these cells the expression level of three genes (IGFBP3, fibronectin and replication protein A) were down-regulated by progesterone. These three genes may therefore be involved in progesterone induced growth regulation of endometrial carcinoma cells. The function of IGFBP3 in cell growth has been studied by other research group and is known to play a putative role in the development of cancer (Giovannucci, 1999). Relatively high plasma IGF-1 and low IGFBP3 levels have been independently associated with a greater risk of cancer development in prostate, breast (in pre-menopausal women), colorectal epithelium and possibly lung. It has been hypothesized that the mitogenic and anti-apoptotic effects of IGF-1 are normally balanced by binding to its major binding protein (IGFBP3). When the levels of IGFBP3 drop, automatically the amount of freely available IGF-1 increases and enhanced cell turnover, with the risk of cellular transformation, may be the result. However, IGFBP3 may also exert growth regulatory effects independently of IGF-1 via its own cellular membrane receptor (Yamanaka et al., 1999). In this way IGFBP3 can induce apoptosis (Baxter, 2001; Butt et al., 1999; Grimberg & Cohen, 1999). Arguing along these lines, progesterone induced down-regulation of IGFBP3 would then result in decreased apoptosis and potentially increased growth.

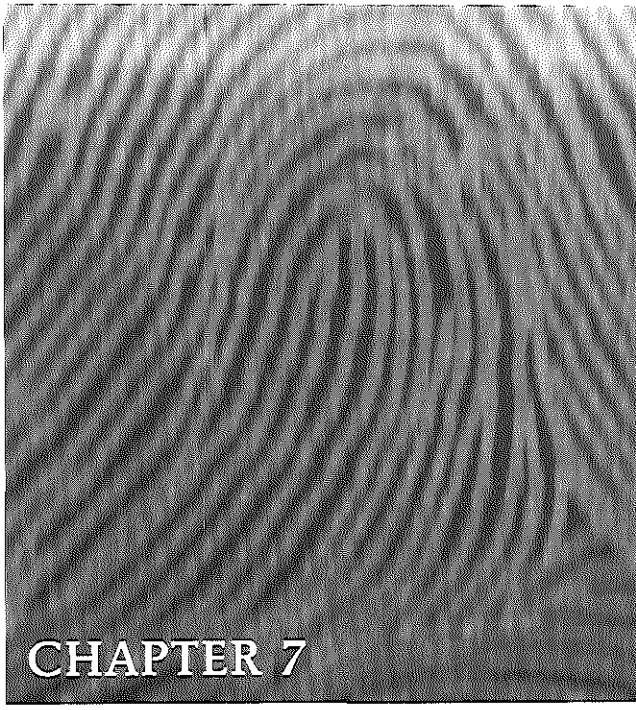
The present investigations, however, do not show an increase in growth with decreased IGFBP3 expression, but rather the opposite. Support for the hypothesis that decreased expression of IGFBP3 may result in growth inhibition comes from research with transgenic animals. Two transgenic models

have been developed for IGFBP3: Neuenschwander et al. (Neuenschwander et al., 1996) reported on a mammary gland targeted IGFBP3 overexpressing mouse model, and Murphy et al. (Murphy et al., 1995) report on a non-tissue specific, inducible IGFBP3 mouse model. When IGFBP3 is overexpressed in the mammary gland, the process of gland-involution after pregnancy is modified by a reduction of apoptosis in transgenic animals (here higher levels of IGFBP3 are correlated with decreased levels of apoptosis). In the mouse model, which harbors inducible IGFBP3 expression, organomegaly was found in the spleen, liver and heart (suggesting that higher levels of IGFBP3 are correlated with increased organ size). It is clear that future research will be necessary to reveal the putative role that IGFBP3 may play in progesterone-induced growth inhibition in the PRB expressing endometrial cancer cell lines.

With the establishment of the two Ishikawa cell lines stably transfected with either hPRA or hPRB, a model to study the possible functional differences of the two hPR isoforms on biological behavior and gene expression of human endometrial cancer cells has been created. Although only a relatively small set of 588 cancer related genes has been examined in this study, distinct differences in progesterone receptor target genes have been found between the two hPR-isoforms.

The present results emphasize the hypothesis that the relative distribution of hPRA and hPRB in endometrial cancer cells may have great implications on human endometrial tumor behavior.

Acknowledgements
We are grateful to Dr. Masato Nishida (Japan) for the gift of the Ishikawa cells, to Dr. B van der Burg (Utrecht, The Netherlands) for the T47D cells, to Dr. E. Milgrom (Paris, France) for providing hPR cDNA and to Dr. S.L.S. Drop (Rotterdam, The Netherlands) for the IGFBP3 cDNA. This work was supported by a grant from The Netherlands Organization for Scientific Research (NWO 903-46-169).



CHAPTER 7

Progesterone regulation of growth and gene expression in human endometrial carcinoma cell lines

Progesterone regulation of growth and gene expression in human endometrial carcinoma cell lines.

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Submitted

Summary

Progesterone plays an important role in controlling proliferation and differentiation of the human endometrium. Using stably transfection techniques, both human progesterone receptor isoforms (hPRA and hPRB) were reintroduced into a well-differentiated human endometrial cancer cell line (a hPR negative sub-clone of the Ishikawa cell line). Several Ishikawa sub-cell lines were constructed each expressing different levels of hPRA, hPRB or both hPRA and hPRB. These Ishikawa sub-cell lines showed a marked progesterone-induced growth inhibition. Upon measuring growth inhibition, it was observed that the sub-cell lines expressing low levels of hPRA were less responsive to progesterone treatment compared to the sub-cell lines expressing similar levels of hPRB. However, cells expressing high levels of the hPR isoforms were all equally growth responsive to progesterone. Using a cDNA microarray, IGFBP3 was shown to be a progesterone regulated gene. The hPRB expressing cells were found to exhibit a more pronounced progesterone induced down regulation of IGFBP3 expression compared to the hPRA expressing cells.

In conclusion, a model system has been generated to investigate progesterone-induced growth inhibition and progesterone-modulated gene expression in human endometrial cancer cells. Furthermore, using this model the distinct functions of the two human progesterone receptor isoforms (hPRA and hPRB) in human endometrial cancer cells can be investigated.

Introduction

Progesterone controls growth of human endometrial epithelium cells by antagonizing the proliferative activity of estradiol and induces differentiation of endometrial epithelium and stromal cells (Martin et al., 1973). Because of its ability to inhibit proliferation and to stimulate differentiation, progesterone has been used to suppress growth of endometrial cancer cells (Ehrlich et al., 1981).

In clinical practice, progesterone is used in a palliative setting as treatment of advanced and recurrent endometrial cancer. However, the response rate of progesterone treatment in these patients is not very high (10-15%) (Lentz, 1994; Rose, 1996). The low response rate is probably due to a transition from hormone-controlled growth towards hormone independent growth in advanced endometrial tumors. In less advanced tumors progesterone treatment can, in theory, be fairly effective. Although surgery is in general curative in these patients and therefore the treatment of choice, progesterone, as a primary treatment, has been used in a small group of pre-menopausal women suffering from well differentiated endometrial cancer and determined to preserve fertility. The response-rate in these patients to treatment with high dose progestins appeared to be in the order of 60% (Kim et al., 1997).

The human progesterone receptor exists as two isoforms, hPRA and hPRB (Horwitz & Alexander, 1983). hPRA and hPRB are not functionally identical. Their relative efficiencies to activate target genes appear to vary according to the promoter and cell context (Meyer et al., 1989). The two isoforms originate from the same hPR gene by transcriptional regulation of two distinct promoters (Kastner et al., 1990). In human target cells both isoforms are coexpressed, generally at comparable levels (Graham & Clarke, 1997). However, the promoters are regulated independently, which can give rise to varying hPRA: hPRB ratio in target tissues under certain physiological circumstances (Kastner et al., 1990; Kraus et al., 1997). The varying ratio of the two isoforms among different target cells and under different physiological circumstances suggests that the differential expression level of PRA and PRB may affect the cellular response to progesterone.

Several research groups have investigated the relative distribution and the putative function of these receptors in the endometrium cells. Using RT-PCR, Fujimoto et al. (Fujimoto et al., 1995) found loss of hPRA in advanced endometrial tumors. Kumar et al. (Kumar et al., 1998) showed that endo-

metrial cancer cell lines only expressed hPRA. Recently Arnett-Mansfield et al. (Arnett-Mansfield et al., 2001) used specific hPRA and hPRB antibodies to study progesterone receptor expression levels in normal and hyperplastic endometrium compared to endometrial cancer. These authors observed that in tumor tissues the staining for progesterone receptors was significantly lower as compared to normal or hyperplastic endometrial tissues. Furthermore, normal glands expressed both progesterone receptor isoforms while in hyperplastic tissue a predominance of either one of the receptor isoforms was observed. In 60% of the tumors either hPRA or hPRB was lost. A few endometrial tumors (10/46) still expressed both progesterone receptor isoforms; interestingly these tumors were all well differentiated tumors (FIGO grade 1). In another study, selective loss of PRB expression, largely due to CpG islands hypermethylation of the PRB promoter region, was observed in 85% of the investigated tumors (Sasaki et al., 2001).

It is difficult to present a clear picture with respect to PR-isoform expression patterns, mainly due to the use of different research materials (cell lines vs. tissues) and/or research methods (RT-PCR [gene transcription] vs. immunohistochemical staining [protein expression]).

In the current investigation, we have used the well-differentiated Ishikawa cell line (Nishida et al., 1996) to generate several hPRA, hPRB and hPRA/hPRB expressing cell lines. It was established that hPR expression in the parental Ishikawa cells was undetectable and addition of progesterone had no effect on growth or gene expression patterns in these parental cells. This hPR-negative sub-clone of the Ishikawa cell line was used to generate hPR expressing cell lines. Eight hPR expressing cell lines were established; expressing hPRA-only, hPRB-only or both progesterone receptor isoforms. These cell lines were characterized in several different ways and provide an accessible tool to investigate progesterone regulated growth inhibition and gene regulation in human endometrial cancer cells.

Materials and Methods

Construction of Ishikawa cell lines stably expressing hPRA, hPRB or both (hPRA and hPRB)

Recombinant Plasmids

The hPR-cDNA cloned into the pSG5 expression vector was a generous gift from Dr. E. Milgrom (Kremlin-Bicetre, Paris, France). This vector was used to create hPRA and hPRB expression plasmids, respectively. A 3063 bp cDNA fragment encoding hPRB was isolated from pSG5-hPR by excision of the *Eco*R1-*Xba*1 fragment (the *Eco*R1 site being artificially introduced at position +14 upstream from the first AUG codon). A 2982 bp cDNA fragment encoding hPRA was isolated by excision of the *Bam*H1-*Xba*1 fragment (the *Bam*H1 site being a hPR restriction site at position - 68 downstream from the first AUG codon and +424 upstream from the second AUG codon). Subsequently, the hPRA and hPRB cDNA fragments were inserted into the pcDNA3.1 expression vector (Invitrogen Corporation, Carlsbad, CA), containing a cytomegalovirus (CMV) promoter and an eucaryotic selection marker. Sequence analysis of the created plasmids was performed to verify proper ligation of the hPR cDNA fragments into the vector. Furthermore, transient transfection experiments in parental Ishikawa cells showed clear transcriptional up regulation of a MMTV-luc construct by both receptor constructs (data not shown).

Cell culture

Ishikawa cells were derived from a well-differentiated human endometrial adenocarcinoma (Nishida et al., 1996) and were a generous gift from Dr. Masato Nishida (Tsukuba, Japan). The cells were negative for mycoplasma contamination, as determined using Mycoplasma-Plus-PCR-Primer-Set (Stratagene, La Jolla, CA). The cells were maintained in DMEM/F12 (Dulbecco's modified Eagle's medium/Ham's F12 (1:1 mix) with 15 mM Hepes, with L-glutamine) supplemented with penicillin/streptomycin; in the presence of 5% fetal calf serum in a 37°C incubator with 5% CO_2. During the experiments, all cell lines were cultured in DMEM/F12 supplemented with 5% dextran coated charcoal treated fetal calf serum, in the presence of penicillin/streptomycin. The cells transfected with either hPRA or hPRB or both were cultured under continuous selection pressure with neomycin (500

μg/ml) and/or hygromycin(250 μg/ml). For total RNA isolation, cells were cultured for the indicated time in the presence or absence of 10^{-7} M Medroxy Progesterone Acetate (MPA, Sigma Chemical co., St Louis, MO). Total RNA was isolated by lyzing the cells with 3M Lithium chloride/ 6M Urea as described by Auffray and Rougeon (Auffray & Rougeon, 1980). The RNA was purified as described by Blok et al. (Blok et al., 1995).

Stably transfection of Ishikawa cell lines with hPRA, hPRB or both (hPRA and hPRB)

Ishikawa cells were plated on 40 cm^2 culture dishes and cultured to 50% confluency. The hPRA-pcDNA3.1+ plasmid, the hPRB-pcDNA3.1+ plasmid and the control "empty-"pcDNA3.1+ vector (Invitrogen Corporation, Carlsbad, CA) were transfected into the Ishikawa cells using FuGENE™ 6 Transfection Reagent (Roche Diagnostics Co. Indianapolis, IN) following instructions provided by the manufacturer. The cells were initially transfected with neomycin selectable hPRA or hPRB constructs, and after several passages, two cell lines were selected. These two cell lines showed highest expression levels of hPRA and hPRB, respectively (cell line PRA-14 and PRB-59). In order to obtain cells expressing hPRA and hPRB, or cells expressing even higher levels of hPRA or hPRB alone, PRA-14 and PRB-59 cells were subsequently transfected with hPRA or hPRB expression vectors selectable for hygromycin.

Characterization of the Ishikawa cell lines stably transfected with hPRA, hPRB or both

^{3}H-R1881-binding assay

The cells were cultured in 6-well plates, under standard conditions as described above, to 75% confluency. The cells were incubated with 8nM ^{3}H-R1881 (NEN Life Science Products, Inc, Boston, MA) in presence or absence of 4μM non-labeled R1881 (NEN Life Science Products, Inc, Boston, MA) for 2 h in a 37°C incubator. The cells were washed five times with phosphate buffered saline (PBS) at 0°C. The cells were lysed in 1M NaOH for 30 min at 60°C. Radioactivity was measured in a liquid scintillation counter and protein concentrations were measured using Bradford's reagent.

Western immuno-blotting

The cells were cultured as described above to 75% confluency. The cells were

washed twice with PBS, lysed in RIPA buffer (40mM Tris-HCL (pH 7.4), 5mM EDTA (pH 8.0), 10% glycerol, 10 mM Sodiumphosphate, 10 mM Sodiummolybdate, 50 mM Sodiumfluoride and 0.5 mM Sodiumorthovanadate 10mM DTT, 1% Triton, 0.08% SDS, 0.5% Deoxycholate, and protease inhibitors: 6 mM PMSF, 5 mM Bacitracin, 5 mM Leupeptin) and centrifuged for 10 min at 350.000 g, 4°C. The proteins were separated on a SDS polyacrylamid gel and transferred to nitrocellulose (Schleicher & Schuell). The hPR (C-20) rabbit polyclonal antibody (Santa Cruz Biotechnology) and human IGFBP3 (C-19) goat polyclonal antibody (Santa Cruz Biotechnology) were incubated with the membrane as follows: The membrane was rinsed with PBS-Tween and blocked for 1 h with blocking solution. The hPR (C-20) antibody was diluted 1:2000 in blocking solution (IGFBP3 diluted 1:4000) and incubated with the membrane for 1 h. The membrane was washed four times 15 min with PBS-Tween. Antibody-peroxidase conjugate was diluted 1:2000 in blocking solution and incubated with the membrane for 1 h. The membrane was washed four times for 15 min with PBS-Tween. The PR and IGFBP3 bands were detected by using Dupont/NEN's Luminol chemiluminescence's procedure and visualized by exposing the blot to X-ray film (Kodak X-Omat, New Haven, CT) for at least 1 min.

Immunohistochemical analysis

The cells were pre-cultured in DMEM/F12 supplemented with 5% stripped fetal calf serum for 3 days before being seeded in collagen gels (collagen kindly provided by Dr. H. Nederbragt, Veterinary Pathology, Utrecht, The Netherlands) at a cell concentration of $2 \cdot 10^6$ cells/ml. 1 ml gels were cultured in 24-well plates for 7 days in DMEM/F12 supplemented with 5% stripped fetal calf serum. The gels were fixed in 3.7% buffered formaldehyde for 24 hours and embedded in paraffin. Sections of the gels were immunohistochemical stained with mouse monoclonal antibodies recognizing PRB specifically (hPRa2) or PRA and PRB both (hPRa8) (both NeoMarkers, Fremont, CA, USA) in a dilution of 1:200. Staining was indirectly visualized using StreptABComplex/ HRP (1:1:200 in PBS) (DAKO, Denmark) and DAB/metal concentrate (Pierce, Rochford, IL, USA).

Progesterone-induced growth inhibition in PRA and/or PRB expressing Ishikawa cells

Cell growth studies

The cells were seeded at 20,000 cells per well in 24-well plates and cultured for the indicated time in the presence or absence of the indicated concentration of Medroxy Progesterone Acetate (MPA, Sigma Chemical Co., St Louis, USA) or R5020 (NEN Life Science Products, INC, Boston, MA) in DMEM/F12 supplemented with 5% dextran-coated charcoal-treated fetal calf serum, and for the transfected cells also supplemented with neomycin at 500 µg/ml and/or hygromycin at 250 µg/ml. The cells were lysed in 150 µl 1M NaOH for 30 min at 60°C and growth differences were obtained by measuring OD 260 values of the lysates.

Analysis of apoptosis

PRAB-36 cells were cultured for 28 or 35 days in the absence or presence of 1 µM or 10 nM MPA. Subsequently the cells were stained with Hoechst 33342 or Propidium Iodide (PI) (Darzynkiewicz et al., 1994). Hoechst 33342 can enter living cells and stains the DNA blue when exposed to 352 nm light. Apoptosis is detected as condensed DNA stains very intense. PI stains the DNA of cells with disrupted cellular membranes (plasma and nuclear membranes), and can therefore be used to detect apoptotic cells. PI dyed DNA stains red when exposed to 530 nm light.

Progesterone regulated gene expression in Ishikawa cells

Atlas Human Cancer cDNA expression array

The Atlas Human Cancer cDNA expression array (7742-1) was purchased from Clontech Laboratories, Inc. (Palo Alto, California, USA). Two identical nucleic acid arrays were supplied containing 588 cancer related human cDNA's spotted as duplicates on nylon membranes. A list of the genes spotted on the array, including array coordinates, is available at Clontech's web site http://www.clontech.com. Total RNA was reverse transcribed into cDNA using a mixture of array gene-specific primers and was labeled with ^{32}P-dATP. Probe purification and hybridization to the array was performed following the array's user manual. The array was exposed to a phosphor-imaging screen at room temperature for 24 hours and scanned using the Phosphor Imager

(Molecular Dynamics, Sunnyvale). The results were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale). A grid was applied to the image of the blot to quantify the intensity of hybridization of every spot. According to the Array manufacturer (Clontech Laboratories, Palo Alto, CA), the radioactive cDNA signal is linear for RNA's present at levels of 0.01-3% of the total RNA population.

Northern-blotting

For total RNA isolation, cells were cultured for 48 hours in the presence or absence of 0.1 μM Medroxy Progesterone Acetate (MPA, Sigma Chemical co., St Louis, MO) in DMEM/F12 supplemented with 7.5% dextran-coated charcoal-treated fetal calf serum, and, for the transfected cells, neomycin at 500 μg/ml and hygromycin at 250 μg/ml. Total RNA was isolated by lyzing the cells with 3M Lithium chloride/ 6M Urea as described by Auffray and Rougeon (Auffray & Rougeon, 1980). The RNA was purified as described by Blok et al. (Blok et al., 1995). Northern blotting was performed as described by Chang et al. (Chang et al., 1997). As a probe, to detect IGFBP3 mRNA on the northern blot, a 2.4 kb EcoR1 cDNA fragment containing the mouse IGFBP3 coding sequence was used. The mouse IGFBP3 sequence has a 80% homology with the human IGFBP3 sequence, and can therefore be used to identify the human IGFBP3 mRNA. This cDNA was kindly provided by Dr. S.L.S. Drop (Department of Pediatrics, division of Endocrinology, Erasmus M.C. Rotterdam, The Netherlands).

Results

Generation of PRA en PRB expressing cell lines

Ishikawa-3H12 cells were obtained from Nishida et al (Nishida et al., 1996). Upon culture for 6 passages these cells were analyzed for progesterone receptor contents. Using specific ligand binding, Northern blotting and western blotting no progesterone receptor levels could be detected (Figure 7.1, lane IK-par, and Smid-Koopman et al., submitted). Subsequently, stably transfections with constructs containing human progesterone receptor A (hPRA) and/or human progesterone receptor B (hPRB) were performed. The cells were initially transfected with neomycin selectable hPRA or hPRB constructs,

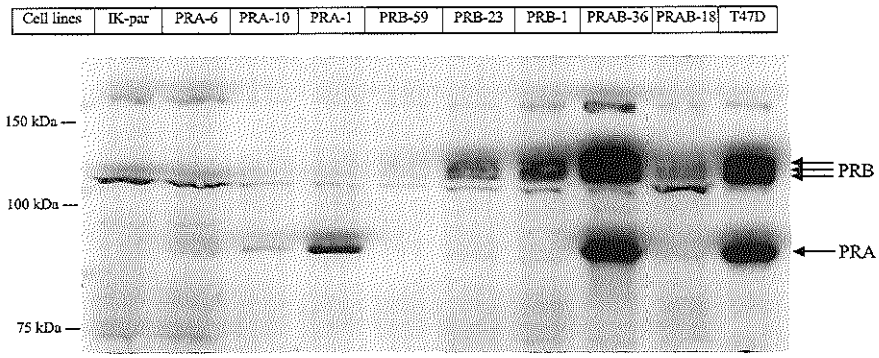


Figure 7.1: PRA and PRB expression in different cell lines. The PRA or PRB expressing cells were cultured under continuous selection pressure with neomycin (500 µg/ml) and/or hygromycin (250 µg/ml) in DMEM/F12 culture medium supplemented with 5% fetal calf serum. Cell lysates were prepared and analyzed on SDS-PAGE. The western blot was incubated with a PR specific polyclonal antibody detecting PRB as well as PRA. Arrows in the margin of the figure indicate the position of PRA and PRB. Molecular weight markers are indicated on the left.

and after several passages, two cell lines were selected (cell line PRA-14 and PRB-59). In order to obtain cells expressing hPRA and hPRB, and cells expressing even higher levels of hPRA or hPRB alone, PRA-14 and PRB-59 cells were subsequently transfected with hPRA or hPRB expression vectors selectable for hygromycin. This lead to the generation of a panel of nine cell lines expressing different amounts of only hPRA (PRA-1, PRA-6, PRA-10, PRA-14), or only hPRB (PRB-1, PRB-23, PRB-59) or both hPRA and hPRB (PRAB-18, PRAB-36). The PRA-6 and PRA-14 cells appeared to express equal amounts of hPRA. Therefore, only one of the two was used for further characterization (PRA-6).

Initially specific radioactive ligand (^{3}H-R1881) binding was used to select progesterone receptor expressing clones. A single-point-binding assay was performed to select clones with a relatively high progesterone receptor content. Subsequently, western blot analysis was used to analyze receptor protein expression and to discriminate between hPRA and hPRB (Figure 7.1).

For the Ishikawa cells expressing high levels of PRA and/or PRB an immunohistochemical analysis was performed. The parental cell line (IK-par), the PRA-1, PRB-1 and PRAB-36 cell lines were cultured in collagen, fixed and stained with a hPRA/B specific antibody or a hPRB specific antibody. It was

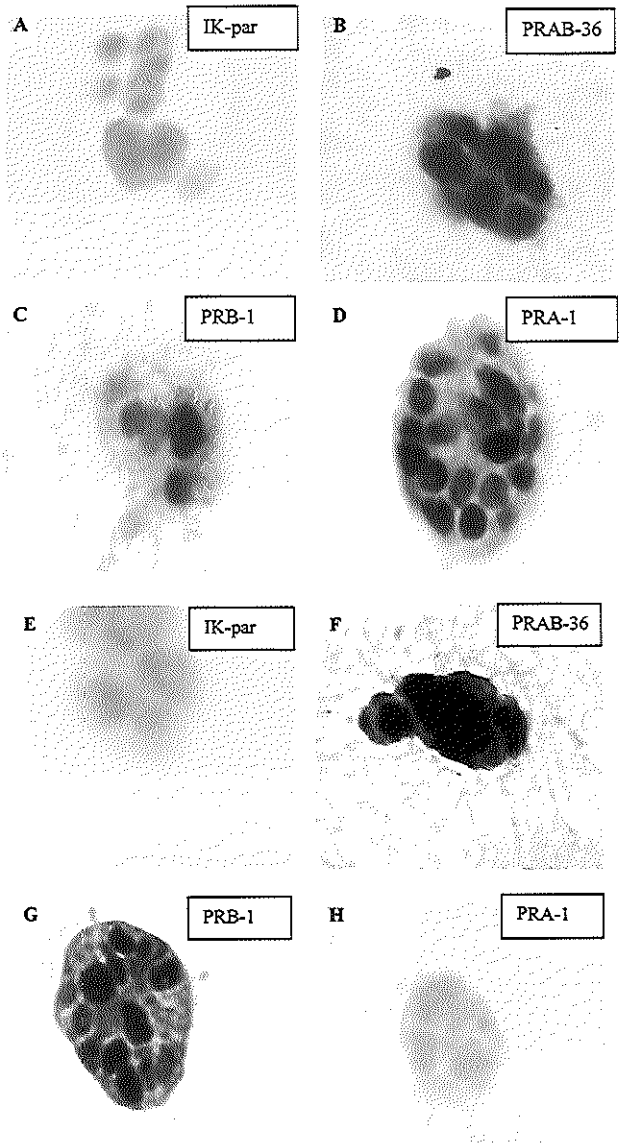


Figure 7.2: Immunodetection of PRA, PRB and PRA/B in stably transfected Ishikawa cells. Parental Ishikawa cells (IK-par), and progesterone receptor containing clones (PRAB-36, PRB-1 and PRA-1) were cultured in collagen, fixed, sectioned and stained using an antibody that detected hPRA and hPRB (Figure 7.2 A-D) or stained with an antibody that detected only hPRB (Figure 7.2 E-H).

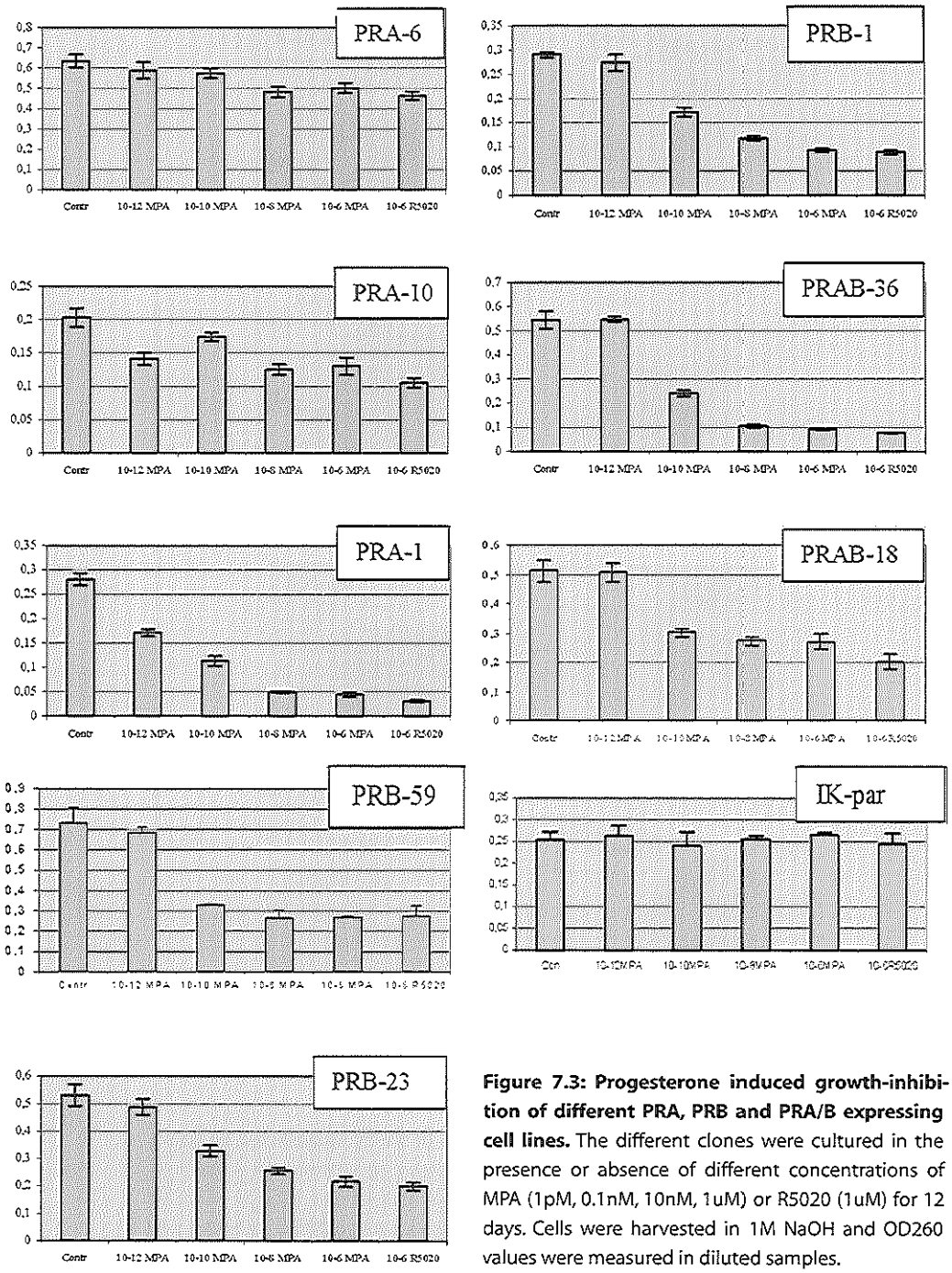


Figure 7.3: Progesterone induced growth-inhibition of different PRA, PRB and PRA/B expressing cell lines. The different clones were cultured in the presence or absence of different concentrations of MPA (1pM, 0.1nM, 10nM, 1uM) or R5020 (1uM) for 12 days. Cells were harvested in 1M NaOH and OD260 values were measured in diluted samples.

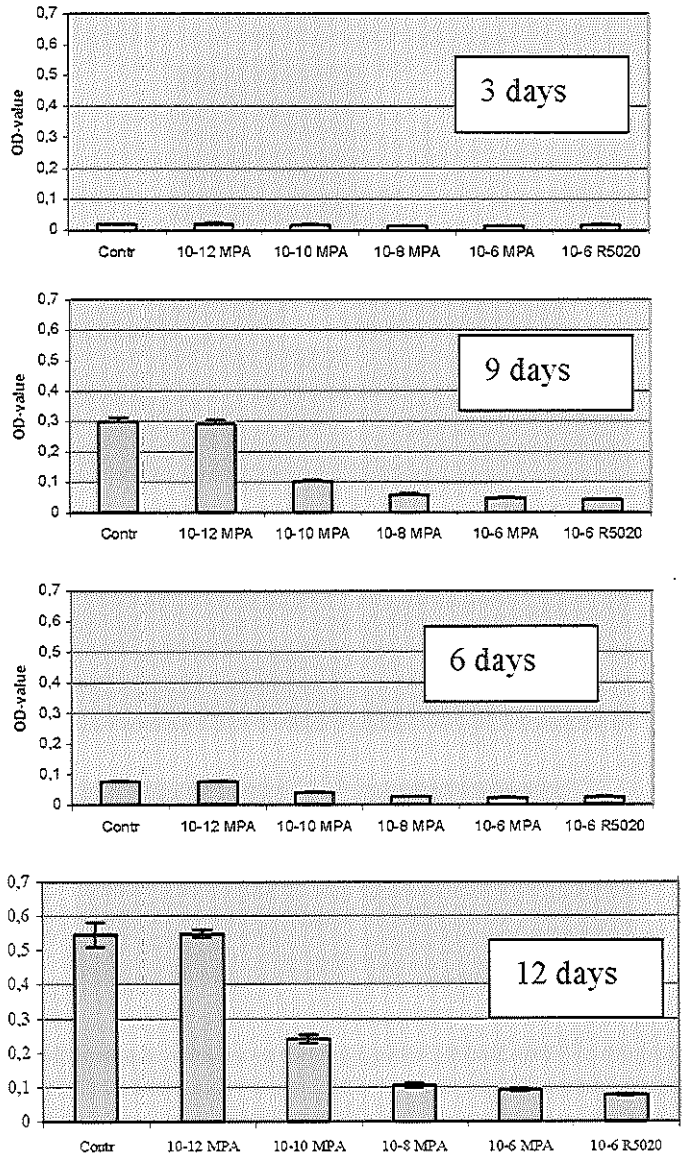


Figure 7.4: Growth inhibition of PRAB-36 cells induced by Medroxyprogesterone Acetate (MPA) or R5020 for different time periods. PRAB-36 cells were cultured for 3, 6, 9 and 12 days in the absence or presence of 1pM, 0.1nM, 10nM or 1μM MPA or 1μM R5020. Cells were harvested in 1M NaOH and OD260 values were measured in diluted samples.

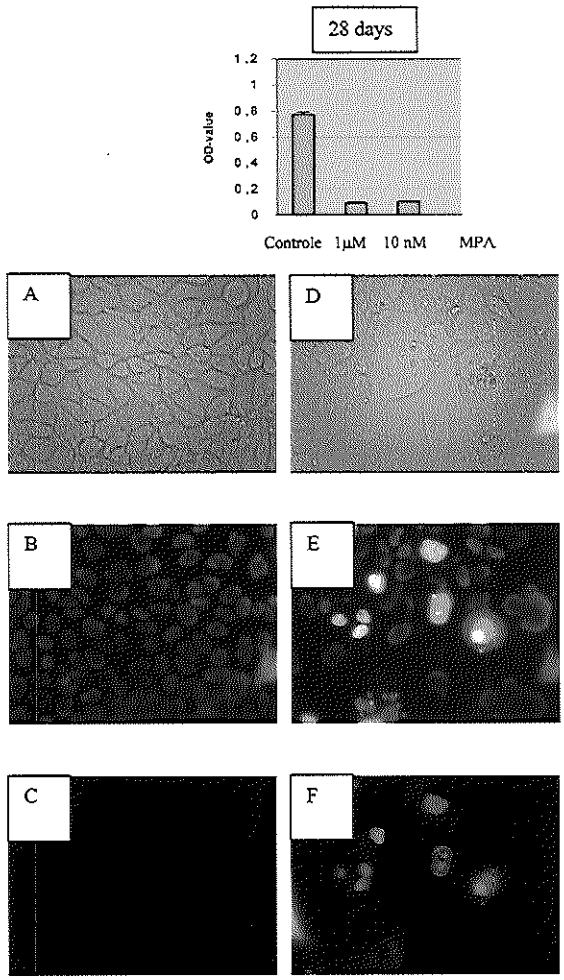


Figure 7.5. Growth of PRAB-36 cells in the presence or absence of progestins for 28 days. PRAB-36 cells were cultured for 28 days in the absence or presence of 10nM or 1µM MPA. For the top figure, cells were harvested in 1M NaOH and OD260 values were measured in diluted samples. Photographs are from control cells (A–C) and cell cultured for 28 days (D–F) in the presence of 1 µM MPA. A and D represent the phase-contrast image of the cells. In the Figures B and E represent the cells were stained with Hoechst 33342 to identify the nuclei. Hoechst 33342 can enter living cells and stains the DNA blue, when exposed to 352 nm light. In the apoptotic cells Hoechst stains the condensed DNA more intense (seen as white). C and F represent cells stained with Propidium Iodine (PI). PI is used to detected apoptosis as it stains the DNA of cells with disrupted cellular membranes (plasma and nuclear membranes) red, when exposed to 530 nm light.

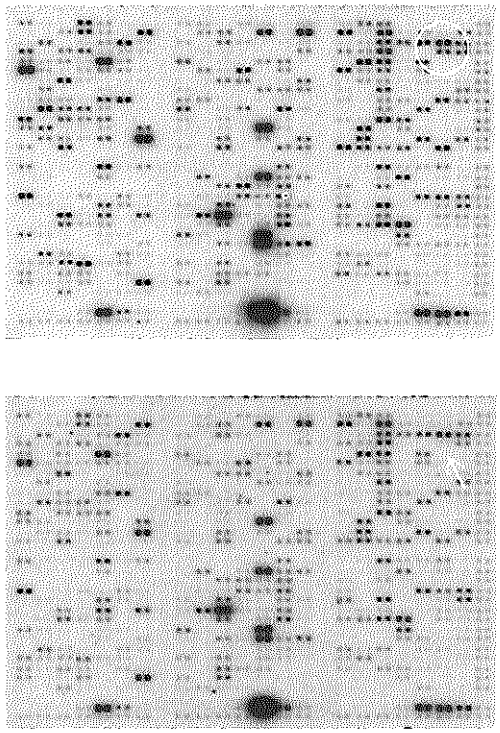
observed that with the hPRA/B antibody hPR staining was detectable in all three cell lines (PRAB-36, PRB-1 and PRA-1) cells (Figure 7.2 B-D). With the hPRB-specific antibody staining was observed only in the cells expressing hPRB (PRAB-36 and PRB-1) (Figure 7.2 F & G) and not in the hPRA expressing cells (PRA-1) (Figure 7.2 H). IK-par cells showed no significant progesterone receptor staining with either antibody (Figure 7.2 A & E).

Progesterone-induced growth inhibition in hPRA and/or hPRB expressing Ishikawa cells

Progesterone treatment of early stage endometrial cancer usually results in growth inhibition of the tumor (Kim et al., 1997). To study these effects of progesterone on endometrial carcinoma cell growth, all isolated hPR expressing clones were cultured in the presence of different concentrations Medroxy Progesterone Acetate (MPA). Growth was measured and it was observed that cells expressing low levels of hPRA (PRA-6 and PRA-10) showed the lowest response to MPA, while in cells expressing comparable levels of hPRB a marked progesterone-induced growth inhibition could be observed. However, all sub-cell lines expressing high levels of hPRA (PRA-1) or hPRB (PRB-1) or hPRA+hPRB (PRAB-36) showed a clear progesterone-induced growth inhibition (Figure 7.3). In order to verify progesterone's ability to induce growth arrest, growth of PRAB-36 cell line was measured after 3, 6, 9 and 12 days in culture in the presence of MPA. It was observed that growth became severely inhibited (Figure 7.4). Moreover, apoptosis could be observed in the PRAB-36 cells when the cells were cultured in the presence of 1uM MPA for a prolonged period of time (28 days) (Figure 7.5).

Progesterone regulated gene expression in Ishikawa cells

To study progesterone regulated gene expression in endometrial carcinoma cells, PRAB-36 cells were cultured in the presence of MPA for 48 h. Subsequently, RNA was isolated and radioactively labeled using RT-PCR. The radioactive probe was used on a cDNA micro-array containing 588 cancer related genes (the Atlas Human Cancer cDNA expression array, Clontech Laboratories, Inc. (Palo Alto, California, USA)). Several genes were found to be regulated by progesterone. The gene with the clearest expression difference was IGFBP3 (Figure 7.6, indicated by the arrow and circle). Because of its



PRAB36 cultured in the absence of medroxy progesterone acetate

PRAB36 cultured in the presence of 0.1 μM medroxy progesterone acetate

Figure 7.6: cDNA microarray of PRAB-36 cells cultured in the presence or absence of 0.1 μM medroxy progesterone acetate. The Atlas Human Cancer cDNA expression array containing 588 cancer related human cDNA's (Clontech Laboratories, Inc. (Palo Alto, California, USA)) was used. A list of the genes spotted on the array, including array coordinates, is available at Clontech's web site http://www.clontech.com. For total RNA isolation, PRAB-36 cells were cultured for 48 hours in the presence or absence of 0.1 μM Medroxy Progesterone Acetate. Probe purification and hybridization to the array was performed following the array's user manual. The array was exposed to a phosphor-imaging screen at room temperature for 24 hours and visualized using the Phosphor Imager (Molecular Dynamics, Sunnyvale).

clear progesterone induced down-regulation, IGFBP3 was chosen for further expression analysis using Northern blotting.

From sub-cell lines cultured for 48h in the presence or absence of MPA mRNA expression of IGFBP3 was measured. Although all hPR expressing cell lines showed down-regulation of IGFBP3 mRNA expression, significant differences in the level of progesterone-induced down-regulation of IGFBP3

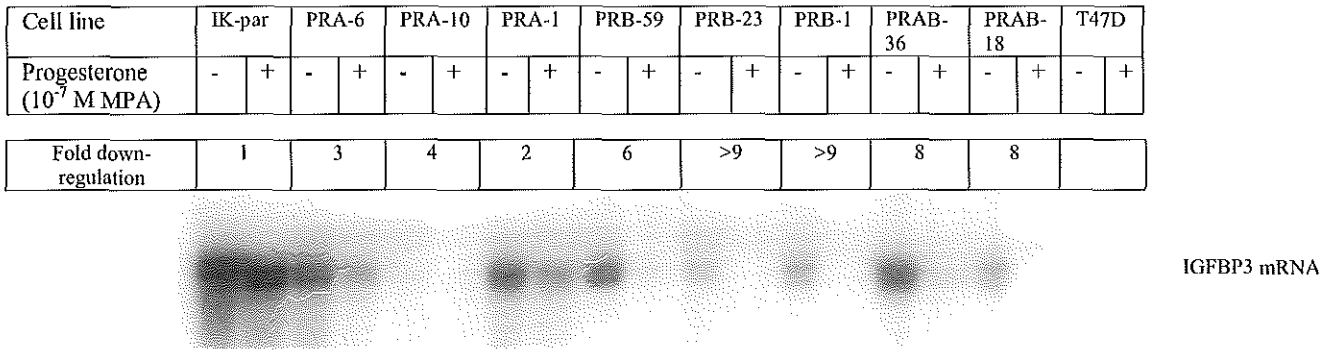


Figure 7.7: Progesterone induced down-regulation of IGFBP3 mRNA. For total RNA isolation, the indicated cells were cultured for 48 hours in the presence or absence of 0.1 μM Medroxy Progesterone Acetate. Northern blotting analysis was performed using a mouse IGFBP3 probe and a hamster β-actin probe. Using a Phosphor Imager the IGFBP3 and actin expression signal level was established. Subsequently, the fold-regulation was calculated.

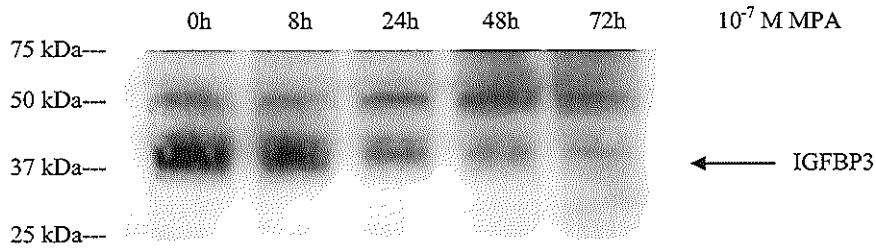


Figure 7.8: IGFBP3 protein is down-regulated by progesterone in the PRAB-36 cell line. PRAB-36 cells were cultured for the indicated time in the presence of 0.1 μM Medroxy Progesterone Acetate. The western blot was incubated with a IGFBP3 specific antibody. Arrows in the margin of the figure indicate PRA and PRB. Molecular weight marker is indicated on the left.

were observed between the two hPR isoforms. The hPRB expressing cell lines (PRB-59, PRB-23, PRB-1, PRAB-36, PRAB-18) showed at least a 6-fold down-regulation of IGFBP3, while the hPRA-only expressing lines (PRA-6, PRA-10 and PRA-1) showed a much more moderate level of progesterone-induced down-regulation of IGFBP3 (3-, 4- and 2-fold, respectively) (Figure. 7.7).

The time-dependency of the progesterone-induced down-regulation of IGFBP3 protein was studied in the PRAB-36 cells. The down-regulation of the IGFBP3 protein concentration appeared to be time-dependent. Marked down-regulation could be observed after 24 hours culturing of the cells in the presence of 0.1 μM MPA (Figure. 7.8).

Discussion

During dedifferentiation of endometrial cancer, progesterone-regulated growth inhibition is often lost. For advanced or recurrent endometrial cancer this limits treatment options. Understanding endometrial cell growth regulation by progesterone may provide tools to improve therapeutic options for patients with advanced or recurrent endometrial cancer.

Dedifferentiation of malignant tumor cells is correlated with changes in expression of several genes. The genes which are known to be frequently mutated or whose expression is otherwise changed in endometrial cancer are

hMLHI (DNA repair) (Parc et al., 2000; Salvesen et al., 2000), PTEN (tumor suppressor) (Kong et al., 1997; Risinger et al., 1997; Tashiro et al., 1997), P53 (tumor suppressor) (Geisler et al., 1999; Ito et al., 1994; Kohler et al., 1996; Silverman et al., 2000), bcl-2 (oncogene) (Ioffe et al., 1998; Zheng et al., 1996) and K-ras (oncogene) (Enomoto et al., 1993; Fujimoto et al., 1993; Lax et al., 2000). Several research groups have studied progesterone receptor expression in endometrial cancer but a clear picture has not emerged from these studies. All studies show reduced expression of the progesterone receptor in a great number of advanced endometrial cancers. However, whether either hPRA or hPRB is selectively lost in endometrial cancer remains debatable. (see Introduction section).

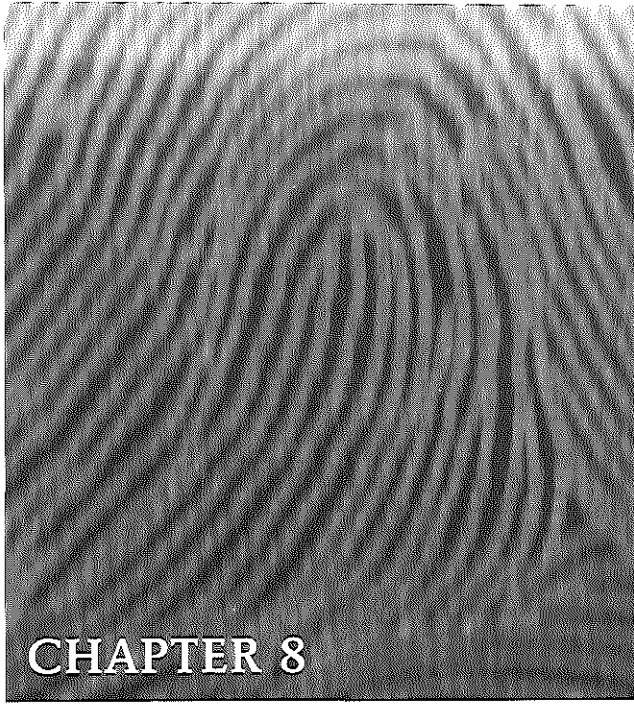
Inhibition of endometrial cell growth by progesterone has been studied recently in Hec50 and KLE endometrial carcinoma cell lines (Dai et al., 2001). These cells were infected with either hPRA or hPRB using adenoviral vectors. A marked effect of progesterone treatment on anchorage independent cell growth was observed in the hPR-infected cells: the hPRA cells showed 50% inhibition of anchorage independent cell growth in response to progesterone treatment, while in hPRB expressing cells 90% inhibition was found. Difference between hPRA-mediated and hPRB-mediated effects on cell growth inhibition could also be observed in the current investigations. Progesterone-induced growth inhibition in cells expressing low levels of hPRA (PRA-6 and PRA-10) was found to be less profound compared to progesterone-induced growth inhibition in cells expressing similar low levels of hPRB (PRB-59 and PRB-23). However, in cells with high expression levels of hPR (PRA-1 and PRB-1), no difference in the growth inhibiting properties of the two different hPR isoforms could be observed. In endometrial cancer it is known that the expression of the progesterone receptor is reduced in a great number of advanced endometrial cancers (Kerner et al., 1995; McCarty et al., 1979; Nyholm et al., 1992). The current results suggest that selective reduction of hPRA expression may have more impact on sensitivity of endometrial carcinoma cells for progesterone-induced-growth inhibition than reduction of hPRB. For clinical practice this could mean that the relative expression level of hPRA may serve as a prognostic marker. Research on human endometrial carcinoma tissue samples combined with clinical outcome will have to be performed to confirm this hypothesis.

In an earlier study by our group (Smid-Koopman et al., submitted) it was also observed that in the hPRA-expressing cell lines different genes were

regulated compared to the hPRB-expressing cell lines. Together with data obtained in other cell systems there is substantial evidence that hPRA expressing cells behave different compared to hPRB or hPRA/B expressing cells (with respect to gene regulation, growth inhibition, anchorage independent growth and metastasis (Graham et al., 1995; Kumar et al., 1998; Mulac-Jericevic et al., 2000; Shyamala et al., 1998)). In endometrial cancer patients there has been observed that losing both progesterone receptor isoforms is associated with dedifferentiation of endometrial cancers (Kerner et al., 1995; Kleine et al., 1990; McCarty et al., 1979; Rakar et al., 1988). However, so far it is not clear what the consequences for cellular functions are upon loss of one or both hPR-isoforms. The currently presented sub-cell lines may provide an accessible tool to investigate this aspect of endometrial cancer development.

One gene (IGFBP3-gene) was shown to be down-regulated by progesterone in two independent cDNA-array experiments. Using Northern and western blotting the progesterone regulation of IGFBP3 could be confirmed. IGFBP3 could be an important gene in cancer development as down-regulation has been correlated with a bad prognosis in ovarian cancer (Katsaros et al., 2002). Furthermore, down-regulation of IGFBP3 is also associated with inhibition of apoptosis (Baxter, 2001; Butt et al., 1999; Grimberg & Cohen, 1999; Shen et al., 1999). The current investigations seem to be in conflict with these reports, as a correlation between progesterone induced down-regulation of cell growth and progesterone induced down-regulation of IGFBP3 expression is observed. However, in a recent publication it was observed that bovine mammary epithelial cells expressing high levels of IGFBP3 were in fact much more sensitive to IGF-induced growth stimulation than cells expressing low levels of IGFBP3 (Grill & Cohick, 2000). Furthermore, in transgenic animals overexpression of IGFBP3 resulted in organomegaly in spleen, liver and heart. From these results it can be extrapolated that progesterone induced reduction of IGFBP3 levels is involved in modulation of endometrial cell growth.

Acknowledgements
We are grateful to Dr. Masato Nishida (Japan) for the gift of the Ishikawa cells, to Dr. E. Milgrom (Paris, France) for providing hPR cDNA and to Dr. S.L.S. Drop (Rotterdam, The Netherlands) for the IGFBP3 cDNA. This work was supported by a grant from The Netherlands Organization for Scientific Research (NWO 903-46-169).



CHAPTER 8

Discussion and Conclusions

8.1 A GENETIC MODEL FOR ENDOMETRIAL TUMORIGENESIS

Today, molecular genetics is an essential part of our growing knowledge and understanding of the etiology and biological behavior of cancer. A review of literature learns that genes most frequently found to be altered in endometrial cancer are the tumor-suppressor genes: p53, PTEN, DCC and the oncogenes: K-ras, c-erbB2, c-fms. Two possible molecular prognostic markers have been identified so far: p53 and c-erbB2 **(Chapter 2.3.3 Table 2.3.1)**.

On basis of histological and clinical characteristics two pathways in endometrial carcinogenesis have been suggested: an estrogen-driven pathway leading to atypical hyperplasia of the endometrium which slowly progresses into an endometrioid carcinoma (Kurman et al., 1985; Lindahl & Willen, 1991) and an estrogen-unrelated pathway leading to the aggressive papillary serous or clear cell carcinomas (Ambros et al., 1995). The available information on the molecular profiles of endometrial cancer seem to support a two pathway theory in the etiology of endometrial cancer: PTEN, K-ras mutations and microsatellite instability are shown to be strongly associated with the prognostic more favorable endometrioid subtype of the endometrial cancers, whereas the aggressive serous carcinomas seem to be associated with p53 and c-erbB mutations. When using this information in combination with Fearons and Vogelsteins model of carcinogenesis (Fearon & Vogelstein, 1990), a two way model for the carcinogenesis of endometrial cancers can be constructed.

However, most endometrial tumors show a genetic profile that only partially fits one of the models presented in Figure 8.1.1. As tumor development seems to be a multi-step process **(Chapter 2.3.3-2.3.4)**, involving many different genetic, epigenetic, and hormonal factors, it seems logical to presume that expression profiles of larger gene clusters may be needed to fully characterize subgroups of endometrial tumors. This has been studied in **Chapter 4** and will be discussed below.

8.2 TUMOR CLASSIFICATION USING cDNA ARRAY TECHNIQUES

Recently, the rapid progression of the Human Genome Project in mapping

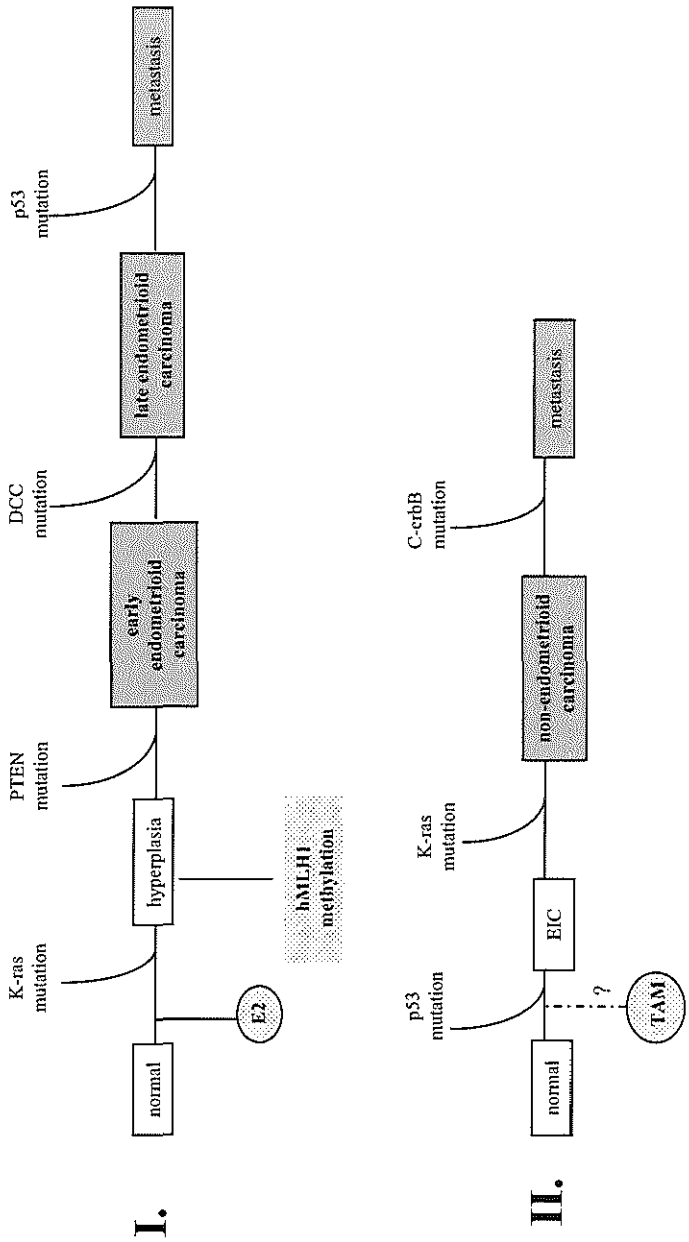

I.
normal
hyperplasia
early endometrioid carcinoma
late endometrioid carcinoma
metastasis
E2
K-ras mutation
PTEN mutation
DCC mutation
p53 mutation
hMLH1 methylation
II.
normal
EIC
non-endometrioid carcinoma
metastasis
TAM
p53 mutation
K-ras mutation
C-erbB mutation
?

Figure 8.1.1: A genetic model for endometrial tumorigenesis. Tumorigenesis is thought to proceed through a series of genetic alterations involving oncogenes and tumor suppressor genes. Endometrial tumors can roughly be divided in two histological sub-types: endometrioid and non-endometrioid. In the etiology of endometrial cancer two pathways have been suggested: an estrogen-driven pathway leading to atypical hyperplasia of the endometrium which slowly progresses into an endometrioid carcinoma (I), and an estrogen-unrelated pathway leading to the aggressive papillary serous or clear cell carcinomas (non-endometrioid carcinoma) via the precursor lesion EIC (endometrial intraepithelial carcinoma) (II). The mutated genes involved in the development of endometrial cancer are indicated. p53 mutations are thought to be an early event in the tumor development in the estrogen unrelated pathway. However, in the estrogen related pathway it seems to occur in a later stage of tumor development and may be responsible for the metastatic property of the tumor. Not only genetic events but also epigenetic events are thought to play a role in endometrial carcinogenesis. Hormonal influences (estradiol and Tamoxifen) and the methylation state have been found to be involved in carcinogenesis.

 = epigenetic events. E2 = estradiol. DCC = deleted in colorectal carcinoma. EIC = endometrial intraepithelial carcinoma.

the human genome and the development of new techniques to study gene expression have created new tools to expand our knowledge of tumorigenesis. The cDNA-array technique has made it possible to study the expression level of thousands of genes in one single experiment. In this way gene expression profiles can be made of every specific tissue type and for every specific condition (e.g. during cell cycle, in response to specific stimuli).

These cDNA array techniques generate a large amount of data, creating a new problem: how to analyze the data. The simplest objective is to compare two distinct condition (e.g. normal cells vs. tumor cells, or treated vs. untreated cells) and identify differentially expressed genes and the degree of similarity between the gene expression profiles. In **Chapter 3** a method is described to compare two gene expression profiles. The most innovative property of this method is the fact that it uses raw (e.g. not normalized) hybridization data. Normalization of the cDNA array data for differences in background signal and for differences in intensity of hybridization is a difficult issue. Local background signal can be subtracted from each hybridization spot, but how to determine local background? Differences in intensity of hybridization can be identified and normalized by using the expression levels of so-called housekeeping genes. However, the expression of housekeeping genes is not guaranteed to be stable in all cell types and during all circumstances. The method described in **Chapter 3** bypasses these problems as preceding normalization of the data for differences in background signal and for differences in intensity of hybridization is not needed.

A higher level of interpreting gene expression profiles is required when the objective is to monitor changes in expression profiles during several steps of a dynamic process, such as tumor development, cell cycle or response to specific stimuli. These experiments require the interpretation of several gene expression profiles simultaneously. Analysis of such massive data sets can only be performed using complex statistical analysis methods. Recently several bioinformatic analysis methods of gene expression data have been established (e.g., Principal components analysis (Hilsenbeck et al., 1999), Boolean genetic networks (Huang, 1999), Self-organizing maps (Tamayo et al., 1999), Hierarchical cluster algorithms (Eisen et al., 1998), Support vector machines (Brown et al., 2000)). Eisen et al. (Eisen et al., 1998) have developed a hierarchical clustering-based algorithm and visualization software package (freely available on http://rana.stanford.edu/software), which is currently one of the most frequently used tools for analyzing gene expression

data. The goal of clustering is to group together objects (genes or samples) with similar properties. Subsequently, several research groups have used hierarchical clustering-based analysis methods on gene expression data obtained from human tissue samples. Iyer et al. (Iyer et al., 1999) used hierarchical cluster analysis on gene expression data from human fibroblasts and found distinct gene clusters each associated with genes with particular biologic functional roles. Alon et al. (Alon et al., 1999) used a two-way clustering method on tumor and normal colon tissues. They found that clustering analysis on basis of gene expression patterns is able to separate cancerous from non-cancerous tissue and cell lines from *in vivo* tissue. Alizadeh et al. (Alizadeh et al., 2000) identified two molecularly distinct forms of diffuse large B-cell lymphoma (DLBCL), which had gene expression patterns indicative of different stages of B-cell differentiation. Moreover, these two groups correlated with patients' survival rates. Also in breast cancer distinct molecular subclasses, correlated with patients' survival rates, have been identified using gene expression profiles and clustering analysis (Perou et al., 2000; Sorlie et al., 2001). Even more, using cDNA array technique and clustering analysis, Scherf et al. (Scherf et al., 2000) found a gene-drug relationship and were able to show that variations in the transcript levels of particular genes relate to mechanisms of drug sensitivity and resistance.

Another method used to analyze gene expression data is supervised clustering of expression data. The goal of supervised analysis is to construct classifiers, which assign predefined classes to a given expression profile. Unsupervised clustering of samples with known characteristics may identify gene clusters, which can subsequently be used as a classifier in supervised analysis of a group of uncharacterized samples. Van 't Veer et al. (van 't Veer et al., 2002) performed such a supervised cluster analysis on gene expression profiles of 117 breast tumors, in order to classify breast tumors according to their clinical behavior. They were able to identify a gene cluster predictive of a short interval to distant metastases. This cluster consisted of genes regulating cell cycle, invasion, metastasis and angiogenesis. They were also able to establish a gene cluster that identifies tumors of BRCA-1 mutation carriers. They predict that gene expression profiling will outperform all currently used clinical parameters in predicting disease outcome and selecting patients for specific therapy.

So far, neither the issue of the diagnostic value of gene expression profiling in endometrial carcinomas nor the feasibility of gene expression profiling

techniques in a clinical setting has been studied. To examine the feasibility of gene expression profiling as a clinical tool, in the current thesis the efficacy, reproducibility and specificity of gene expression profiles of human endometrial carcinoma tissues obtained from surgical specimen was studied (Chapter 4). The efficacy to generate a gene expression profile of a tumor appeared to be mainly determined by the efficacy of the tissue sampling from the surgical specimen. Tissue sampling was performed guided by macroscopic judgment of the tissue. Most likely the efficacy can be improved when the sampling would be performed on basis of microscopic judgment of fresh frozen slides of the sample.

In this thesis a commercial cDNA array is used to generate gene expression profiles from endometrial carcinoma tissue samples (Atlas Human Cancer cDNA Expression Array, Clontech, USA). The array contains 588 genes. The genes are selected by the manufacturer on basis of their known involvement in cancer development. It includes oncogenes, tumor suppressor genes, cytokines and other genes related with cell cycle regulation, growth regulation, apoptosis, DNA repair, cell adhesion and motility, angiogenesis, invasion regulation or cell-cell interaction. It was hypothesized that these genes, on basis of their known functional roles in tumorigenesis, could serve as a classifier in human cancers. To study the diagnostic and prognostic value of gene expression profiling in human endometrial carcinoma tissue samples, this cDNA array was used and subsequently a supervised cluster analysis of the obtained gene expression data was performed (Chapter 4). Large-scale gene expression monitoring to select a classifier was not possible, as at the time, large scale gene expression monitoring was not yet available in research laboratories in the Netherlands and too expensive to obtain commercially. Eisen's software for a hierarchical clustering-based algorithm (http://rana.stanford.edu/software) seemed promising in providing an useful tool to analyze large masses of gene expression data (Alizadeh et al., 2000; Eisen et al., 1998; Perou et al., 1999; Perou et al., 2000; Ross et al., 2000; Scherf et al., 2000; Sorlie et al., 2001). Moreover, it is supplemented with a software package that visualizes the clustering results and is freely available on the Internet. Using Eisen's software, supervised analysis was performed and two distinct endometrial cancer clusters were found. It was not possible to identify a smaller group of genes within the array genes, with a higher specificity as classifier. The two endometrial tumor clusters highly resembled the FIGO grading system. However, whether gene expressing profiling will outperform

all currently used clinical parameters in predicting disease outcome and selecting patients for specific therapy, as hypothesized by van 't Veer et al. (van 't Veer et al., 2002), is not yet possible to determine.

8.3 Progesterone-induced growth inhibition of endometrial cancer cells

In the endometrium, the growth inducing properties of estradiol and the growth inhibiting properties of progesterone coexist in a delicate balance. In endometrial carcinomas this fine balance may be lost. Subsequently, due to loss of progesterone-sensitivity during tumor progression, the response rate of hormonal treatment in patients with advanced or recurrent endometrial cancer is low (10-15%). For the treatment of advanced or recurrent endometrial cancer this creates a problem, as treatment options are limited in these tumors. Understanding of the regulation of endometrial cell growth inhibition by progesterone may provide tools to improve therapeutic options for patients with advanced or recurrent endometrial cancer.

The human progesterone receptor exists as two isoforms, hPRA and hPRB. The relative distribution of these two isoforms varies in target tissues and under certain physiological circumstances (Kastner et al., 1990; Kraus et al., 1997). In the endometrium, the hPRA predominates in stromal cells throughout the menstrual cycle, whereas in epithelial cells, a shift occurs during the early secretory phase from hPRA to hPRB (Mote et al., 1999). The varying ratio of the two isoforms among different target cells and different physiological circumstances suggests divergent putative function for these two receptors.

Using stable transfection techniques, the human progesterone receptor was reintroduced into a hPR negative sub-clone of the Ishikawa endometrial carcinoma cell line. Either one or both progesterone receptor isoforms (hPRA and hPRB) were transfected. In this way several Ishikawa sub-cell lines were constructed, each expressing different levels of hPRA, hPRB or hPRA and hPRB, respectively. These Ishikawa sub-cell lines provide a new model to study the possible functional differences of these two receptors. All cell lines were characterized with regard to progesterone induced growth response and progesterone regulated gene expression. Remarkable differences were found between the cells expressing different hPR isoforms. Upon measuring growth

inhibition, it was observed that the sub-cell lines expressing low levels of hPRA were less responsive to progesterone treatment compared to the sub-cell lines expressing similar levels of hPRB or hPRA/hPRB. However, cells expressing high levels of the hPR isoforms were all equal growth responsive to progesterone **(Chapter 7)**. Differences in expression of genes targeted by the two isoforms were studied using cDNA expression array technique. A different set of genes appeared to be progesterone regulated in the hPRA cells compared to the hPRB cells **(Chapter 6)**.

These observations support the hypothesis of distinct functional differences between the two progesterone receptor isoforms. Moreover, these results seem to implicate that relative loss of expression of the B isoform is involved in the loss of progesterone induced growth inhibition in endometrial cancers. Subsequently, this could mean that tumors with relative loss of hPRB expression have a worse prognosis compared to tumors showing high hPRB expression levels. Several studies support this hypothesis. Kumar et al.(Kumar et al., 1998) showed that poorly differentiated endometrial cancer cell lines expressed hPRA only. Sasaki et al. (Sasaki et al., 2001) investigated the CpG hypermethylation status of human endometrial cancer cell lines and patient tissues, and observed selective loss of PRB expression, largely due to hypermethylation, in 85% of the investigated tumors (Sasaki et al., 2001). Dai D et al. (Dai et al., 2001) infected endometrial carcinoma cell lines (KLE and Hec 50) with adenoviral vectors encoding the genes for progesterone receptor isoforms A and B. They found that expression of the progesterone receptor isoform B caused a much more dramatic decrease in cell growth compared to expression of the progesterone receptor isoform A. In breast tumor similar results were found. Shyamala et al. (Shyamala et al., 1998) found that in transgenic mice overexpressing PRA, the mammary gland exhibits ductal hyperplasia with a disorganized basement membrane and decreased cell-cell adhesion; features that are commonly associated with neoplasia. Other studies contradict the hypothesis that PRB is predominantly responsible for the progesterone induced cell growthinhibition. Using PRA knockout (PRAKO) mice, Mulac-Jericevic et al (Mulac-Jericevic et al., 2000) showed that progesterone treatment of these animals resulted in PRB dependent increase in proliferation of endometrial cells of these PRAKO-mice. The conflicting results may be due to differences in research models: human (present thesis, (Dai et al., 2001)) vs. mice (Mulac-Jericevic et al., 2000).

Knowledge of the relative distribution of hPRA and hPRB in human en-

dometrial carcinoma tissues combined with clinical outcome or FIGO stage would be informative for the clinical implications of loss of one of the hPR-isoforms in endometrial tumors. However, this has not been studied very extensively yet. There is one report in the literature, using RT-PCR and based on a very low number of patients, which seems to point to a more predominant appearance of hPRB in more advanced endometrial tumors (Fujimoto et al., 1995). Arnett-Mansfield et al (Arnett-Mansfield et al., 2001) used specific hPRA and hPRB antibodies to study progesterone receptor expression levels in normal and hyperplastic endometrium compared to endometrial cancer. They observed that in tumor tissues the staining for progesterone receptors was significantly lower as compared to normal or hyperplastic endometrial tissues. Furthermore, normal glands expressed both of the progesterone receptor isoforms while in hyperplastic tissue a predominance of one of the receptor isoforms was observed and in 60% of the tumors either hPRA or hPRB was lost. However, no relation was found between the isoforms lost and the differentiation grade of tumor. In breast cancer, Graham et al. (Graham et al., 1995) found low levels of hPRB in a subset of the tumors, however neither histological grade nor clinical outcome were provided.

Identifying genes involved in the downstream cascade following progesterone binding to its receptor may help to understand the molecular mechanism behind progesterone-induced growth-inhibition of endometrial cancer cells and provide tools to restore the progesterone-induced growth inhibition of hormone resistant endometrial cancer. In **Chapter 5** a differential display PCR method (ddPCR) was used to identify progesterone-regulated genes in endometrial carcinoma cells. Only genes progesterone-regulated in the cells growth responsive to progesterone (Ishikawa endometrial carcinoma cells (Nishida et al., 1996)) and not regulated in the cells not growth responsive to progesterone (ECC-1 endometrial carcinoma cells) were selected. Genes selected in this way could be genes exclusively involved in progesterone-induced growth inhibition. One EST and three known genes (dsRNA adenosine deaminase, ninein, endothelin converting enzyme-1) matched these criteria. Further characterization of the clones, with respect to their involvement in progesterone-induced growth-inhibition of endometrial cancer cells, was not performed. The Ishikawa cells appeared to lose their progesterone receptor already after few cell culture passages, making further characterization of the clones difficult. All other Ishikawa sub-cell lines tested, were not stable in their expression of the progesterone receptor. Subsequently, new Ishi-

kawa sub-cell lines had to be established, exhibiting stable expression of the progesterone receptor. As mentioned earlier, using stably transfection techniques, the human progesterone receptor was reintroduced into a hPR negative sub-cell line of the Ishikawa cell line. In this way several new Ishikawa sub-cell lines were created, which showed a marked progesterone-induced growth-inhibition **(Chapters 6 and 7)**.

The rapid progression of the development of techniques to study gene expression is reflected in this thesis. At the start of the research for this thesis, ddPCR was a frequently used method to study differential gene expression **(Chapter 5)**. However, only a few years later the ddPCR method had been replaced by the cDNA array techniques. Subsequently, when the new progesterone receptor expressing Ishikawa cell lines were established, differences in gene expression were studied using cDNA arrays **(Chapters 6 and 7)**.

Using cDNA array technique, IGFBP3 was identified as a progesterone down-regulated gene in all Ishikawa sub-cell lines **(Chapter 7)**. Reports on the possible role of IGFBP3 in tumor behavior are conflicting. Down-regulation of IGFBP3 has been associated with inhibition of apoptosis (Baxter, 2001; Butt et al., 1999; Grimberg & Cohen, 1999; Shen et al., 1999) and with worst prognosis in ovarian cancer (Katsaros et al., 2002). These observations suggest that IGFBP3 expression is associated with inhibition of cell growth. However, in the current investigations **(Chapter 7)** the hPRB expressing cells were found to exhibit a more pronounced progesterone induced down regulation of IGFBP3 expression compared to the hPRA expressing cells. This suggests a correlation between progesterone-induced down-regulation of cell growth and progesterone induced down regulation of IGFBP3 expression. A recent publication of Grill and Cohick (Grill & Cohick, 2000) supports these findings. They stably transfected bovine mammary epithelial cells with IGFBP3 and observed that high IGFBP3 levels were correlated with an increased IGF-induced cell growth rate. Even more, in transgenic animals a correlation was found between high levels of IGFBP3 and increased cell growth: overexpression of IGFBP3 resulted in organomegaly of spleen, liver and heart.

The current results emphasize distinct functional differences for the two progesterone receptor isoforms in human endometrial carcinoma cells. The two isoforms regulate different sub-sets of genes. At relatively low expression levels, hPRB seem to be more effective in inhibiting cell growth. Whether interaction between the isoforms is important in generating progesterone

actions in target tissue is unknown. On basis of the observations described in this thesis it can be hypothesized that the relative distribution of hPRA and hPRB in endometrial cancer cells has great implications on human endometrial tumor behavior. Still, the specific action of two isoforms on progesterone target tissues such as endometrial cells remains to be clarified.

8.4 Conclusions

This thesis has concerned itself with the possible role of molecular genetics in improvement of the outcome of endometrial cancer patients. In this respect four questions were assigned. This paragraph addresses the answers obtained with the studies performed in the thesis.

1. *What degree of clinical utility and subsequent diagnostic value of gene expression profiling of endometrial tumors can be ascertained?*
   Using a commercially available cDNA array technique, gene expression profiles can be generated from human endometrial carcinoma tissue samples with high efficacy (77%). The gene expression profiles seem to be tissue specific and not patient specific. As a result of these observations, it can be concluded that gene expression profiling seems to be a clinical feasible tool.
   Ranking endometrial tumors on basis of their gene expression profiling showed high similarity with FIGO grading, indicating that the diagnostic value of gene expression profiling matches the FIGO grading system.

2. *Is it possible to identify genes that are differential expressed between progesterone-sensitive and progesterone-insensitive endometrial cancer cells?*
   Using differential display PCR technique four genes have been identified to be differential expressed between progesterone-sensitive and progesterone-insensitive endometrial cancer cells: dsRNA adenosine deaminase, ninein, endothelin converting enzyme-1 and one EST. These four genes may be involved in the development of progesterone insensitivity in endometrial cancers.

3. *What functional differences between the two human progesterone receptor (hPR) isoforms (hPRA and hPRB) in endometrial cancer cells can be established?*
   Distinct differences in progesterone regulated gene expression and in progesterone induced cell growth inhibition were observed between hPRA and hPRB expressing endometrial cancer cells.

4. *What effects of progesterone can be measured on gene expression and cell growth of endometrial cancer cell lines expressing hPRA or hPRB or both?*
   With regard to cell growth inhibition, endometrial cancer cells expressing relative low levels of hPRA are found to be less responsive to progesterone treatment compared to cells expressing similar levels of hPRB. However, cells expressing relative high levels of one or both hPR isoforms are equally growth responsive to progesterone. Upon gene expression regulation, progesterone is found to regulate a different set of genes in hPRA expressing cells compared to hPRB expressing cells.

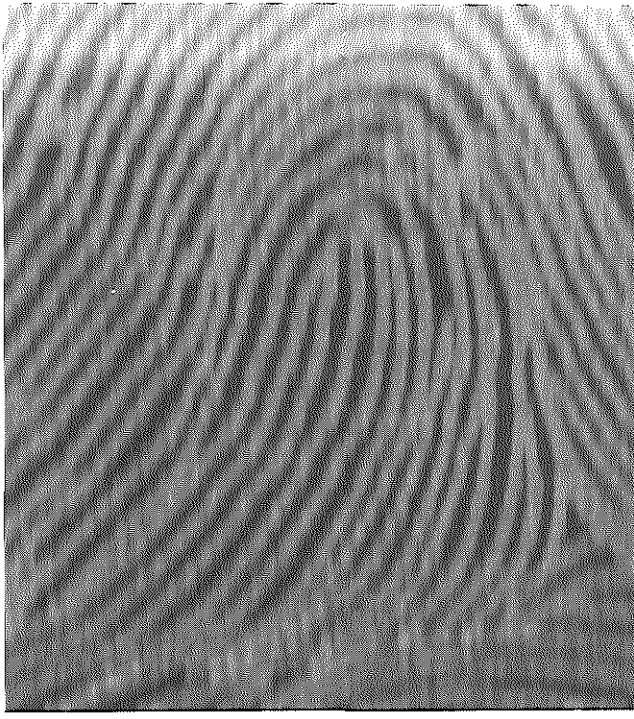
8.5 Future directions

To determine whether gene expressing profiling will outperform all currently used clinical parameters (e.g. FIGO staging system) in predicting disease outcome and selecting patients for specific therapy, as hypothesized by van 't Veer et al. (van 't Veer et al., 2002), a larger group of endometrial tumor gene expression profiles will have to be combined with clinical long term follow up data. Unsupervised clustering of genome wide expression data may help to select a more specific cluster of genes to use as classifier in supervised clustering. Insertion of genes, identified to be involved in progesterone induced growthinhibition, in the classifier cluster may help to subtract sub-groups of patients responsive to hormonal treatment.

Studying the relative distribution of hPRA and hPRB expression in the human endometrial cancer tissue samples and subsequently combining these data with long term clinical follow-up may help to establish the prognostic implication of selective loss of one of the progesterone receptor isoforms.

To illuminate the specific functions of the hPRA and hPRB in endometrial cancer cells, the recent established Ishikawa sub-cell lines provide a useful model. The functional role of the five progesterone-regulated genes (one EST,

dsRNA adenosine deaminase, ninein, endothelin converting enzyme-1 and IGFBP3) will have to be further characterized in these cells.



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Summary

In the Netherlands approximately 370 women die of endometrial cancer every year. Part of these deaths is accounted for by patients who were misguidedly taken to have a good prognosis as they were classified in the low surgical-pathological stage (stage 1 and 2) group (FIGO staging system). The other part of the deaths is accounted for by patients with advanced disease. In these patients the response rates to treatment (surgery combined with radiotherapy) is low (26% five years survival rate). Hormone therapy (e.g. progesterone) is given to these patients as a palliative treatment. However, due to loss of progesterone sensitivity during tumor progression, the response rate of hormonal treatment in these patients is also low (10-15%).

Genetic alterations are thought to play a major role in tumor development and tumor progression. The recent rapid progression of the Human Genome Project in mapping the human genome and the development of new techniques to study gene expression have created new tools to expand our knowledge of tumorigenesis. Several genes have been identified to be associated with tumor development in human tissues. Recently, it has been shown that gene expression profiling can be used to identify distinct tumor subclasses, which correlate with the biological behavior of the tumor, and which were previously undetected by the surgical and histological classification methods.

In this thesis the possible role of molecular genetics in improvement of the outcome of endometrial cancer patients is investigated. To improve tumor classification the value of gene expression profiling in classifying human endometrial tumors is studied. To find tools to restore the progesterone-induced growth inhibition of endometrial cancer, the molecular mechanisms involved in progesterone-induced growth inhibition of endometrial cancer cells are studied.

In **Chapter 2.1** an overview is given of the characteristics and the biological behavior of normal human endometrium and of human endometrial cancer. **Chapter 2.2** describes the mechanism of action and functional characteristics of the human progesterone receptors (hPRA and hPRB). The General Introduction ends with a review of literature studying genetic events in endometrial cancer **(Chapter 2.3)**. Genes most frequently found to be altered

in endometrial cancer are the tumor-suppressor genes: p53, PTEN, DCC and the oncogenes: K-ras, c-erbB2, c-fms. Two possible molecular prognostic markers have been identified so far: p53 and c-erbB2.

In **Chapter 3** the Atlas Human Cancer cDNA expression array is used to generate gene expression profiles of human endometrial cancer and benign endometrial tissue samples. Subsequently, a method to compare two gene expression profiles was introduced. The most innovative property of this analysis method is the fact that it uses raw hybridization data; normalization of the data for differences in background signal and for differences in intensity of hybridization is not needed. Three genes (Decorin, TIMP3 and Cyclin D1) are identified to be differentially expressed between benign endometrial and endometrial carcinoma tissue. Decorin and TIMP3 are down-regulated and Cyclin D1 is up regulated in tumor tissue samples.

In **Chapter 4** the utility and diagnostic value of gene expression profiling in human tissue samples is studied. The Atlas Human Cancer cDNA array containing 588 cancer related genes is used to generate gene expression profiles of hysterectomy specimen. The efficacy to generate a gene expression profile of a tumor appears to be mainly determined by the efficacy of the tissue sampling from the surgical specimen. A clustering analysis is performed. Two distinct endometrial cancer clusters are found on the basis of their gene expression profile. These two clusters highly resemble classification according to the FIGO grading system.

In **Chapter 5** four cDNA clones are identified to be progesterone regulated and differential expressed between a progesterone sensitive and a progesterone insensitive endometrial carcinoma cell line. The cDNA clones are identified using a differential display PCR method. Sequence analysis reveals that three cDNA clones match with known genes (dsRNA adenosine deaminase, ninein, endothelin converting enzyme-1).

Chapters 6 and 7 describe the establishment of several new endometrial carcinoma cell lines. Using stably transfection techniques, the human progesterone receptors (hPRA and hPRB) are reintroduced into a hPR negative sub-clone of the Ishikawa endometrial carcinoma cell line. In this way several Ishikawa sub-cell lines are constructed, each expressing different levels of hPRA, hPRB or hPRA and hPRB. These Ishikawa sub-cell lines provide a new model to study the possible functional differences of these two receptor isoforms. All cell lines are characterized with regard to progesterone induced growth response and progesterone regulated gene expression. In **Chapter 6**

differences in gene expression between hPRA and hPRB are studied using two of the newly created Ishikawa cell lines (PRA-14 and PRB-59). A different set of genes appears to be progesterone regulated in the PRA-14 cells compared to the PRB-59 cells. None of the genes are regulated by both hPRA and hPRB. **Chapter 7** describes the establishment and characterization of nine Ishikawa sub-cell lines (four expressing hPRA, three expressing hPRB and two expressing hPRA + hPRB). Remarkable differences in progesterone induced growth response are found between the cells expression different hPR isoforms. The sub-cell lines expressing low levels of hPRA appear to be less responsive to progesterone treatment compared to the sub-cell lines expressing similar levels of hPRB or hPRA/hPRB. Using the Atlas Human Cancer cDNA array progesterone regulated genes are identified in a hPRA and hPRB expressing Ishikawa sub-cell line (PRAB-36). Several genes are found to be regulated by progesterone. The gene with the most significant expression difference is IGFBP3. IGFBP3 is down-regulated by progesterone. IGFBP3 mRNA appears to be progesterone regulated in all Ishikawa sub-cell lines. However, all hPRB expressing cell lines show a more profound down-regulation of IGFBP3 compared to the hPRA expressing lines.

In the Discussion and Conclusions section **(Chapter 8)** a genetic model for endometrial tumorigenesis is presented. The prognostic/diagnostic value of gene expression profiling of endometrial tumor samples is discussed. Furthermore, distinct functional differences between the two hPR isoforms in endometrial cancer cells are discussed. Finally, suggestions are given concerning possible future directions of the research related to this thesis.

SAMENVATTING

In Nederland sterven jaarlijks ongeveer 370 vrouwen ten gevolge van endometriumkanker. Voor een deel betreft dit vrouwen die aanvankelijk, met behulp van het chirurgisch/histologische FIGO stageringssysteem, geclassificeerd waren in een prognostisch gunstige groep (FIGO Stadium 1 en 2). Het andere deel van de sterfgevallen betreft patiënten met een gemetastaseerde tumor. Deze patiënten worden, indien mogelijk, behandeld met chirurgische verwijdering van de tumor gecombineerd met bestralingstherapie. De genezingskans voor deze vrouwen is laag (vijfjaars overleving slechts 26%). Hormonale therapie (progesteron) kan als een vorm van palliatieve behandeling gegeven worden. Echter, tijdens het voortschrijden van de tumorontwikkeling blijken endometrium tumorcellen hun gevoeligheid voor progesteron te verliezen. Door dit verlies aan progesterongevoeligheid is de kans op succes van de palliatieve therapie dan ook laag (bij slechts 10-15% van de patiënten wordt een verbetering waargenomen).

Aan genetische veranderingen wordt een grote rol toebedeeld in de ontwikkeling en progressie van humane tumoren. Het Human Genome Project heeft recent grote vooruitgang geboekt in het ontcijferen van het humane genoom. Dit heeft, samen met de ontwikkeling van nieuwe onderzoekstechnieken, gezorgd voor nieuwe middelen om onze kennis van kanker uit te breiden. Van verschillen genen is betrokkenheid bij de ontwikkeling van kanker vastgesteld. Bovendien is recentelijk ontdekt dat met behulp van gen expressie profielen, nieuwe tumor subklassen aan te wijzen zijn. Deze nieuwe subklassen correleren met specifiek biologisch gedrag van de tumoren. De voorheen gebruikelijke chirurgisch/histologische stageringssystemen zijn niet in staat deze subklassen te onderscheiden.

In dit proefschrift is de rol die de moleculaire genetica zou kunnen spelen in het verbeteren van de overleving van vrouwen met endometriumkanker onderzocht. De overleving van deze patiënten zou verbeterd kunnen worden door optimalisatie van de tumorclassificatie. Moleculaire classificatie middels gen expressie profielen zou een verbetering van deze classificatie kunnen geven. In dit proefschrift is de diagnostische waarde van moleculaire classificatie middels gen expressie profielen in endometriumtumoren bestudeerd .

Daarnaast zijn in dit proefschrift de moleculaire mechanismen betrokken bij progesterongeïnduceerde groeiremming van endometriumcellen onderzocht. Inzicht in deze mechanismen zou kunnen helpen om middelen

te vinden waarmee de progesteron geïnduceerde groeiremming in endometrium tumoren kan worden hersteld. Hiermee zouden nieuwe therapeutische mogelijkheden voor patiënten met endometrium kanker ontwikkeld kunnen worden.

In de Algemene Introductie (**Hoofdstuk 2.1**) wordt een overzicht gegeven van de karakteristieken en het biologisch gedrag van normaal endometrium weefsel en van endometriumtumoren. **Hoofdstuk 2.2** beschrijft het functioneren van de humane progesteron receptoren (hPRA and hPRB). Tevens wordt een overzicht van de literatuur betreffende moleculair genetische veranderingen in endometrium kanker gegeven (**Hoofdstuk 2.3**). In endometriumtumoren worden veranderingen in expressie niveau het meest frequent gevonden voor de tumor-suppressor genen: p53, PTEN en DCC en voor de oncogenen: K-ras, c-erbB2, c-fms. Van twee genen (p53 en c-erbB2) blijkt dat zij als prognostische markers kunnen fungeren. De moleculair genetische informatie die tot nu toe over endometriumkanker beschikbaar is, ondersteunt de theorie dat in de etiologie van endometriumkanker twee subgroepen aan te wijzen zijn: 1) een oestrogeengerelateerde etiologie waarbij veranderingen in PTEN, K-ras en microsatelliet instabiliteit betrokken zijn (het endometrioid subtype), 2) een niet oestrogeengerelateerde etiologie welke leidt tot de meer agressieve papillair sereuse en clear cell tumoren (non-endometrioid subtype), en waarbij p53 en c-erbB2 mutaties betrokken lijken te zijn.

In **Hoofdstuk 3** wordt beschreven hoe met behulp van een "cDNA array" techniek, gen expressie profielen gemaakt worden van endometriumtumor weefsel en van normaal endometriumweefsel. Vervolgens wordt een methode beschreven om gen expressie profielen van twee verschillen weefsels te vergelijken. Het meest innovatieve aan deze nieuwe analyse methode is het feit dat gebruik gemaakt wordt van onbewerkte hybridisatie data; normalisatie van de data voor verschillen in achtergrondsignaal of voor verschillen in intensiteit van de hybridisatie is niet nodig. Van drie genen (Decorin, TIMP3 en Cycline D1) is aangetoond dat zij differentieel tot expressie komen tussen normaal endometrium weefsel en endometriumkankerweefsels. Het expressieniveau van Decorin en TIMP3 is laag in de tumorweefsels in vergelijking tot het normale weefsel. De expressie van Cycline D1 daarentegen is hoog in tumorweefsel en laag in het normale (niet-tumor) weefsel

In **Hoofdstuk 4** wordt de bruikbaarheid en de diagnostische waarde van gen expressie profilering in humane weefsels bestudeerd. Een cDNA array,

welke 588 kanker gerelateerde genen bevat, is gebruikt om gen expressie profielen te generen uit hysterectomie materiaal. Een clustering-analyse van de verkregen data is verricht. Twee endometriumkanker clusters worden op basis van verschillen in gen expressie profielen geïdentificeerd. De verdeling van de tumoren over deze twee clusters blijkt grote gelijkenis te vertonen met de verdeling van de tumoren op basis van het FIGO graderingssysteem.

In **Hoofdstuk 5** zijn vier progesterongereguleerde cDNA's geïdentificeerd, welke differentieel tot expressie komen tussen een voor progesteron groeigevoelige endometriumcarcinoom cellijn en een voor progesteron groeiongevoelige endometriumcarcinoom cellijn. De cDNA's zijn geïdentificeerd met behulp van een differential display PCR methode. Sequence analyse laat zien dat drie van deze cDNA's overeenkomen met reeds bekende genen (dsRNA adenosine deaminase, ninein, endothelin converting enzyme-1).

In de **Hoofdstukken 6 en 7** wordt de ontwikkeling van een groep nieuwe endometriumcarcinoom cellijnen beschreven. Met behulp van stabiele transfectietechnieken werden de humane progesteronreceptoren (hPRA and hPRB) getransfecteerd in een hPR negatieve subkloon van de Ishikawa endometrium carcinoom cellijn. Hiermee zijn verschillende nieuwe Ishikawa subcellijnen gecreëerd. Deze subcellijnen vertonen elk een verschillend expressie niveau van hPRA, hPRB of hPRA en hPRB. Met deze cellijnen is de mogelijkheid gecreëerd om de functionele verschillen tussen de twee progesteron receptor isovormen te kunnen bestuderen. Alle cellijnen zijn gekarakteriseerd met betrekking tot progesterongeïnduceerde groeiremming en progesteron gereguleerde gen expressie. In **Hoofdstuk 6** zijn twee van de nieuwe Ishikawa cellijnen (PRA-14 en PRB-59) gebruikt om verschillen in gen expressie profiel tussen hPRA en hPRB te bestuderen. In de PRA-14 cellen blijken andere genen door progesteron gereguleerd te worden dan in de PRB-59 cellen. Er worden geen genen gevonden die zowel in de PRA-14 als in de PRB-59 cellen door progesteron gereguleerd worden. In **Hoofdstuk 7** wordt de ontwikkeling en karakterisering van negen Ishikawa subcellijnen (vier hPRA, drie hPRB en twee hPRA + hPRB) beschreven. Tussen de hPRA en de hPRB bevattende cellijnen worden opmerkelijke verschillen gevonden in gevoeligheid voor progesterongereguleerde groeiremming. De subcellijnen met lage expressieniveaus van hPRA blijken minder gevoelig voor progesteron gereguleerde groeiremming dan de cellijnen met vergelijkbare niveaus van hPRB of hPRA en hPRB expressie. Met behulp van cDNA array techniek zijn progesterongereguleerde genen geïdentificeerd in een hPRA en hPRB bevattende Ishikawa subcellijn

(PRAB-36). Verschillende progesterongereguleerde genen zijn geïdentificeerd. IGFPB3 is het gen met het meest duidelijke progesteron gereguleerde expressie niveau. Het expressieniveau van IGFBP3 daalt na toediening van progesteron. Northern blot analyse is gebruikt om het expressieniveau van IGFBP3 te bestuderen in de hPRA en de hPRB bevattende Ishikawa cellijnen. IGFBP3 blijkt progesterongereguleerd in alle Ishikawa subcellijnen. Echter, de hPRB cellijnen laten een meer uitgesproken regulatie van IGFBP3 expressie zien dan de hPRA cellijnen.

In **Hoofdstuk 8** wordt een moleculair-genetisch model voor endometrium carcinogenese gepresenteerd. Vervolgens wordt de prognostische en diagnostische waarde van tumor gen expressie profielen bediscussieerd. Tevens worden de functionele verschillen tussen de twee hPR isovormen besproken. De in dit proefschrift gevonden resultaten worden geïnterpreteerd in het licht van de beschikbare literatuur over dit onderwerp. Ten slotte worden suggesties gedaan ten aanzien van mogelijk vervolgonderzoek.

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Curriculum vitae

The author of this thesis was born on the 10th of October 1966 in Hilversum. In 1985 she passed her secondary school exam (VWO-B) at the Chr. Atheneum Adriaen Pauw in Heemstede. From 1985 to 1993 she attended the Medical School at the Free University (VU) in Amsterdam. Subsequently, she started to work as a resident (AGNIO). At first from August 1993 to March 1995 at the IJsselmeer Hospital in Lelystad (at the departments: Gynecology & Obstetrics, Surgery, Internal Medicine and the ER). Followed by a residency (AGNIO) Obstetrics & Gynecology at the Reinier de Graaf Hospital in Delft from March 1995 to June 1996. In June 1996 she started to work at the Erasmus M.C. Rotterdam as a resident (AGNIO), again at the department of Obstetrics & Gynecology (head: Prof. dr Th.J.M.Helmerhorst).

During her AGNIO residency period at the Erasmus M.C. Rotterdam the research described in this thesis was performed. The research started March 1997 and was performed at the laboratory of dr. A. O. Brinkmann at the department of Reproduction & Development of the Erasmus M.C. Rotterdam (head: Prof. dr. J.A. Grootegoed). The research was supervised by Prof. dr. Th. J. M. Helmerhorst, dr. F. J. M. Huikeshoven and dr. A. O. Brinkmann. In January 2001 she started her AGIO residency Obstetrics & Gynecology at Saint Clara Hospital (MCRZ) in Rotterdam (head: dr. J. A. Wijnen). She is married to Edzko Smid. In November 2001 their son Meint was born.